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Population Genetics and Immune Response to Staphylococcus pseudintermedius

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To the Graduate Council:

I am submitting herewith a dissertation written by Samar Mohammed Mansour Solyman entitled "Population Genetics and Immune Response to Staphylococcus pseudintermedius." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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Population Genetics and Immune Response to *Staphylococcus pseudintermedius*

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Dedication

This Doctoral dissertation is dedicated to my father and my mother

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Abstract

Staphylococcus pseudintermedius is an opportunistic bacterium affecting canines that has recently developed a high prevalence of methicillin resistance and corresponding multi-drug resistance. Developments of alternatives to antibiotics such as vaccines are important strategies for control of this organism.

Multilocus sequence typing is based on the sequence variations of slowly evolving genes and usually uses a minimum of seven genes. Population genetic studies of *S. pseudintermedius* have been based on sequencing four genes (MLST-4). Forty-five genes were selected from available genomic data and tested as MLST candidates. Four genes were amplified from all isolates and showed the highest number of alleles. By adding four new genes to the existing ones, an MLST scheme based on 8 genes (MLST-8) was developed for *S. pseudintermedius.* MLST-8 was applied to 176 isolates of previously characterized *S. pseudintermedius* from dogs (165 isolates), cats (5 isolates) and humans (6 isolates). They were contained in 83 sequence types (STs). MLST-8 identified 106 STs which indicated a high level of diversity of the species but also verified the clonal nature of methicillin resistant isolates.

Five different STs of *S. pseudintermedius* were selected and reacted with serum samples from dogs with pyoderma and healthy control dogs to measure their immune reactivity. Significant differences in the IgG reactivity between the different genetic backgrounds of *S. pseudintermedius* were found, this variation should be considered in designing a vaccine for *S. pseudintermedius.* The reactivity of other staphylococcal species with canine pyoderma and healthy control sera showed that minor genetic differences may be associated with significant variations in IgG reactivity.

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List of Abbreviations

agrD – accessory gene regulator, encodes AIP and serves as gene loci for Bannoehr et al. MLST

AIP – auto-inducing peptide

ATCC – American Type Culture Collection

clfA - Staphylococcal fibrinogen-binding protein

CoNS – coagulase-negative staphylococci

cpn60 – chaperonin 60, gene loci for Bannoehr et al. MLST

CWA - cell wall-associated proteins

ELISA - Enzyme-linked immunosorbent assay

fdh – Formate dehydrogenase

HA-MRSA – hospital-acquired methicillin-resistant *Staphylococcus aureus*

HGT – horizontal gene transfer

IgG – Immunoglobulin G

mecA – gene encoding penicillin-binding protein 2α

MGE – mobile genetic element

MLST – multilocus sequence typing

MLVA – multiple-locus variable-number tandem-repeat analysis

MRSA – methicillin-resistant *Staphylococcus aureus*

MRSP – methicillin-resistant *Staphylococcus pseudintermedius*

MSSA – methicillin-susceptible *Staphylococcus aureus*

MSSP – methicillin-susceptible *Staphylococcus pseudintermedius*

PBP2a – penicillin-binding protein 2a

PFGE – pulsed-field gel electrophoresis

pta – phosphate acetyltransferase gene, gene loci for Bannoehr et al. MLST

pfoR - Perfringolysin O regulatory protein

purA – Adenylosuccinate synthetase

sar - Sodium: sulfate symporter

sdr D - Staphylococcal fibrinogen-binding protein

SIG – *S. intermedius* group (*S. delphini*, *S. intermedius*, and *S. pseudintermedius*)

ST – sequence type, refers to MLST

sodA – superoxide dismutase

spa – staphylococcal protein a

sps D – Cell wall associated protein in *S. pseudintermedius* mediates the binding to cytokeratin 10

tuf – elongation factor tu, gene loci for Bannoehr et al. MLST

UTCVM – University of Tennessee College of Veterinary Medicine

Chapter 1: Literature Review

Overview

The development of a staphylococcal vaccine is a challenging task in part due to genetic heterogeneity of the species. Broad understanding of the nature of the immune response to staphylococcal infections as well as correlation between genetic differences and pathogenesis might facilitate this task. *Staphylococcus pseudintermedius* is an important opportunist bacterium that frequently causes diseases in dogs and occasionally causes zoonotic infections to human beings [1-4]. It is commonly associated with canine pyoderma [5-7] and is also frequently associated with urinary tract, wound and surgical site infections [8-11]. Methicillin resistance in *S. pseudintermedius* has been reported with high prevalence in the last few years [7, 12-20]. Approximately 30% of the *S. pseudintermedius* isolates tested by the University of Tennessee College of Veterinary Medicine (UTCVM) Clinical Bacteriology Laboratory were methicillin-resistant [19, 20]. The importance of methicillin-resistant *S. pseudintermedius* (MRSP) in veterinary medicine compares to the importance of methicillin-resistant *S. aureus* (MRSA) in human medicine.

S. pseudintermedius is a coagulase-positive bacterium that was first reported as a distinct species in 2005 [10]. It was originally identified as *Staphylococcus intermedius* prior to classification as a member of the *S. intermedius* group (SIG) [21], a group consisting of *S. intermedius, S. delphini* and *S. pseudintermedius.* The isolates were phenotypically similar to *Staphylococcus intermedius* and *Staphylococcus delphini* and *16s rRNA* gene sequence analysis revealed that they have 99% similarity [10].The three species differentiated by using DNA–DNA hybridizations, *S. pseudintermedius* strains had binding values of 38–46% with *S. intermedius* LMG 13351 and 54% with *S. delphini* DSM 20771. It was subsequently determined that most isolates phenotypically identified as *S. intermedius* from dogs were *S. pseudintermedius* [7, 22]. The genome of *S. pseudintermedius* is composed of a single circular chromosome ranging from 2,572,216 bp to 2,617,381 bp and has an average G+C content of 37.6%, which is substantially higher than the 32% average of other staphylococci [23, 24].

Humoral immunity is an important arm of the acquired branch of the immune system in protection against extracellular bacterial infections. However, its role in immunity against staphylococcal infections is unclear. Some anti-staphylococcal antibody levels are associated with immunity to specific staphylococcal infections

such as antibodies to the staphylococcal exoprotein TSST-1[25]. On the other hand, heterogeneous antibody responses have been found in both healthy individuals and patients with different *S. aureus* infections [26]. No commercial vaccines are available for staphylococcal infections. Immunity to *S. pseudintermedius* infections is poorly understood. In addition, the population genetics of *S. pseudintermedius* is not well characterized and there is a dearth of information relating antigenic variation with genetic background.

S. pseudintermedius as a human pathogen

S. pseudintermedius is considered a true pathogen of pet animals such as dogs and cats. Considering the close social contacts between human and pet animals, it may cause diseases in human beings. *S. pseudintermedius* is occasionally isolated from healthy or infected human beings [2, 3, 27-30]. The occurrence of *S. pseudintermedius* in human infections is probably underestimated, because in some laboratories all coagulase-positive staphylococci are classified as *S. aureus* [31]. The first reported case of the isolation of *S. intermedius* from a human was in 1997 from a nasal swab from a woman with chronic renal insufficiency with no signs of infection [4]. Noninvasive zoonotic infection of *S. intermedius* was reported in [32], where *S. intermedius* was isolated from the ear fluid of a patient with otitis externa. Analysis of 16S ribosomal DNA restriction fragment length polymorphisms identified that *S. intermedius* in the patient and his pet dog were similar which indicated noninvasive zoonosis.

S. pseudintermedius infection in human was reported in 2006 for an ischemic cardiomyopathy patient who received an implantable cardioverter-defibrillator (ICD). Infection in the ICD was suspected and the infected ICD was surgically removed. *S. pseudintermedius* was isolated from the samples taken from the ICD. The source of the isolate was not known nor is it known if the patient kept any pets [2]. Zoonotic potential of S. *pseudintermedius* was studied by Hanselman et al [33], *S. pseudintermedius* nasal colonization was in 4% of sampled humans and it was isolated from 46% of dogs. Indistinguishable strains of *S. pseudintermedius* were found in 44% of households where both a dog and human were colonized which supports canine to human transmission [33]. A confirmed case of catheter-related bacteremia due to *Staphylococcus pseudintermedius* in a child with dog exposure was also reported [34].

Methicillin-resistant *S. pseudintermedius* (MRSP) with a specific genetic background known to be widely disseminated in European countries (sequence type 71) was isolated from a human infection [28]. The prevalence of colonization of MRSP among small animal dermatologists attending a national veterinary conference was reported recently in Italy [35] where MRSP was found in 7 out of 128 veterinarians. The sampling was from nasal swabs and no methicillin-susceptible *S. pseudintermedius* (MSSP) was found. A follow-up study of two carriers revealed that MRSP persisted for at least 1 month in the nasal cavity. These results highlight the possibility of the transmission of *S. pseudintermedius* between pets and humans.

The emergence of methicillin resistance in *S. pseudintermedius*

Resistance to methicillin is mainly caused by the acquisition of new resistance genes and the mechanism by which this occurs is as yet unknown. The *mecA* gene encodes a penicillin-binding protein with reduced affinity for methicillin [17, 36]. The *mecA* gene is contained within mobile genetic elements known as Staphylococcal Chromosome Cassette mec (SCCmec) elements [37]. In total, at least seven different SCCmec classes have been described for *S. aureus*, defined by the content of the regulatory gene region, the allotype of the recombinase genes ccrA and ccrB, and by the overall genetic structure [38]. SCCmec can be transferred between different staphylococcal species in vivo [39].In this report *mecA* positive methicillin-resistant *S aureus* (MRSA) of a certain pulsed-field gel electrophoresis genotype were isolated from a neonate who had never been in contact with MRSA. This MRSA contained *mecA* DNA with an identical PFGE profile to that in a coagulase-negative Staphylococcal strain isolated from the same patient, but different from other MRSA genotypes. These results suggest that the MRSA was formed in vivo by horizontal transfer of the *mecA* DNA between two staphylococcal species. The same mechanism may be found in *S. pseudintermedius*, however, information about the SCCmec donor as well as the genetic specifications of the SCCmec recipient is lacking.

S. pseudintermedius SCCmec elements SCCmec II-III and SCCmec IV had previously been found in two different geographical locations. The first report of methicillin resistance in *S. pseudintermedius* confirmed by PCR detection of *mecA* occurred in 1999 from a single isolate in a survey of 25 staphylococcal isolates of canine origin [36]. Reported isolations of methicillin-resistant *S. pseudintermedius (*MRSP) have increased significantly in the past few years [7,

12-20]. Methicillin resistance in *S. pseudintermedius* is considered a significant problem worldwide because of multi-drug resistance and due to the limited number of drug choices remaining to treat serious infections caused by these organisms.

Little is known about the spread of antibiotic resistance in *S. pseudintermedius* including rates of horizontal gene transfer. A recent study examined the antibiotic susceptibilities of 39 *mecA* positive isolates of *Staphylococcus intermedius* from animals, staff and environment in an animal hospital and determined the possibility of horizontal transmission between animals and humans. Similar genetic finger prints were observed between seven isolates from an animal, two isolates from veterinary staff, and the environment in one animal hospital, and single isolates from an animal and a veterinarian at another hospital which suggests the possibility of horizontal transmission of *S. pseudintermedius* containing *mecA* between humans, animals, and the environment in animal hospitals [40].

Phenotypic and molecular Identification of *S. pseudintermedius*

Differentiation between members of the SIG by phenotypic tests is difficult. *S. intermedius* can be differentiated from *S. pseudintermedius* by a combination of biochemical tests (arginine dihydrolase production, ß-gentiobiose fermentatio and D-mannitol fermentation). However, biochemical reactions do not reliably distinguish between *S. pseudintermedius* and *S. delphini* [22]. Commercial identification systems for the fast and correct identification of *S. pseudintermedius* are not available to date. *S. pseudintermedius* is a relatively newly recognized species and remains to be included in the databases of most systems.

Biochemical markers of canine coagulase-positive staphylococci (CoPS) such as acetoin production, assimilation of maltose, galactose, trehalose, lactose and mannitol broth fermentation were evaluated recently by Chanchaithong [41] for the identification of *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans* and *S. aureus* . They were successfully used to distinguish the three species of canine CoPS from other CoPS species. The results were compared with multiplex-PCR (M-PCR) and there was a good level of agreement with the result of M-PCR. Species-specific protein patterns were useful for phenotypic differentiation of staphylococcal species.

In many cases isolates may be misidentified as *S. intermedius* or *S. aureus* [2]. In a case study reporting a postoperative sinusitis, a methicillin-resistant *S. pseudintermedius* was initially misidentified as MRSA because the identification as *S. aureus* was only based on a positive tube coagulase test [42]. The isolate was re-identified as *S. intermedius* but this isolate was most likely *S. pseudintermedius* because the source of the isolate was a dog.

Molecular identification of *S. pseudintermedius* was first described in 2007 [21] based on partial *sodA* gene sequences and *hsp60* gene sequences. Phylogenetic analysis was sufficiently discriminative for *S. intermedius* and *S. pseudintermedius.* In 2009 a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method was developed based on *MboI* restriction of *pta* gene fragment [43]. This method is considered highly valuable especially when used with biochemical testing and sequencing of housekeeping genes. However, it misclassified a small fraction of the *S. pseudintermedius* population as a result of heterogeneity in the *MboI* restriction sites [44].

Genetic Typing of *S. pseudintermedius*

The recent emergence of methicillin- resistant strains has increased the need for a reliable method for typing *S. pseudintermedius*. Bacterial typing is an essential application in modern microbiology studies for several reasons: enhancing outbreak investigations and surveillance, studies of the natural history of infection, and our understanding of the transmission, pathogenesis and phylogeny of bacteria. There are no standard protocols for molecular typing of *S. pseudintermedius* to date.

Typing methods applied to *S. pseudintermedius* include staphylococcus protein A (*spa*) typing [45], pulsed field gel electrophoresis (PFGE) [7, 45, 46] and multilocus sequence typing (MLST) [7]. No one technique is optimal for all types of investigations. The choice of the typing method is based on the application or the research objectives.

Pulsed-Field Gel Electrophoresis

PFGE is a highly discriminatory whole genome typing method. It involves the use of restriction enzymes with few restriction sites to generate a limited number of high molecular-weight restriction fragments, depending on the number and location of restriction sites within the genome. These fragments are then

separated by agarose gel electrophoresis with programmed variations in both the direction and the duration of the pulsed field [47, 48]. The resulting electrophoretic patterns are highly specific for strains from a variety of organisms and also provide an opportunity to examine multiple variations throughout the genome of the organism so as to identify specific strains and accurately link them with disease outbreaks. Because the frequency of point mutations that can add or delete restriction sites is high, band patterns change relatively quickly through successive bacterial generations. Thus, PFGE is more useful in comparing isolates from a limited area over a short period of time such as isolates from a single hospital in an outbreak situation [6, 49, 50]. The main disadvantage of PFGE is the poor standardization for inter-laboratory comparison as the banding patterns vary greatly depending upon the agarose gel, power supply settings, and analysis software used. Attempts to solve this problem with *S. aureus* have been made [47]. PFGE is not suitable for long-term epidemiological surveillance and it cannot be used for discrimination between members of the SIG group.

Multilocus Variable Number Tandem Repeat Analysis (MLVA)

MLVA is a PCR method based on the analysis of the variation in the number of repeats in several genes. The MLVA method was developed for *S. aureus* in 2003 based on seven genes *(sspA, spa, sdrC, sdrD, sdrE, clfA,* and *clfB*) [51]. In MRSA studies MLVA was found to be comparable in discriminatory power with PFGE [52-55].
The minimal requirements for MLVA ensure low setup and operation costs, which may make this method a simple substitution for PFGE. Until now, MLVA has not been developed for *S. pseudintermedius* typing.

Tandem repeat sequence analysis of staphylococcal protein A *(spa)*

spa typing is a single-locus PCR based typing method based on tandem repeat sequence analysis of a highly polymorphic region of staphylococcal protein A (*spa*) gene. The *spa* typing protocol was first developed for *S. aureus* in 1996 [56]. The study showed that the X-region of the protein A gene is sufficiently stable for epidemiological typing of MRSA and it is more sensitive than phage typing. The polymorphic region of the *spa* gene is classified based on small repeats and point mutations. Because it is a single locus typing method, spa typing may require validation by another method such as PFGE. The *spa* locus of

S. pseudintermedius was characterized [45] and is used for typing MRSP isolates together with other methods such as PFGE and MLST [49, 57] It is a simple, less time-consuming method than PFGE, and results of *spa*-typing can be compared between laboratories.

Multi locus sequence typing (MLST)

Multilocus sequence typing (MLST) was first developed in 1998 [58, 59] as a general approach for typing bacteria. MLST is based on the sequence variation of internal fragments of about 450–500 bp from selected housekeeping genes. For each locus, the different sequences are assigned as alleles, and alleles at all sequenced loci provide a profile of an isolate which defines the sequence type. A distinct sequence type (ST) is made up of a unique combination of arbitrarily numbered alleles [60-65]. Measurement of bacterial genomic variation through time and over great distances is the main application of MLST. This method has been developed for diverse species of bacteria and generally uses at least seven loci [58, 61, 62, 66, 67]. The main advantage of MLST is its use of sequence data that is fully portable and can be stored in a single expanding central multilocus sequence database. It can be interrogated electronically via the Internet to produce a powerful resource for global epidemiology. MLST data can be used for the identification of lineages that have an increased propensity to cause disease as well as tracking of the genetic lineages with the highest levels of antibiotic resistance.

MLST for *S. aureus* was developed using seven loci [62] and *S. aureus* STs are assigned by comparing sequence data for each locus to the MLST data base at http://saureus.mlst.net/. Application of MLST in a collection of blood isolates resulted in two major clusters, one containing only MSSA and the other only MRSA. These clusters are closely related (differing at only one of the seven loci), which suggests that the MRSA clone might have arisen from an MSSA clone that was associated with serious disease [61].

A four gene loci MLST for SIG was developed. Housekeeping genes that have been used as targets in MLST of *S. pseudintermedius* include the *16S rRNA* gene, heat shock protein gene (*cpn60*), elongation factor gene (*tuf*) phosphate acetyltransferase gene (*pta*) and accessory gene regulatory (*agrD*) [7]. A minimum of seven genes are generally included in MLST schemes. Due to lack of sequence variations in conserved genes, it is unusual for an MLST scheme to include the *16S rRNA* gene [7]. The addition of new genes to the existing *S.*

pseudintermedius MLST scheme will increase the discriminatory power equivalent to MLST used for other species in order to obtain better knowledge of *S. pseudintermedius* population genetics.
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Staphylococcal Cassette Chromosome (SCCmec) Typing

The large mobile genetic element (MGE), SCCmec, is characterize by the presence of two genetic components, mec complex which is responsible for the expression and the regulation of *mecA* gene (IS431mec, *mecA* gene, intact or truncated sets of regulatory genes *mecR1* and *mecI)* and the *ccr* complex which encodes recombinase genes (ccr) which mediate the integration of SCCmec into and its excision from the recipient chromosome [68]. SCCmec typing can be used only for typing resistant isolates. There are at least five classes of *mecA* genes (A, B, C1, C2, and E) based on the sequence and completeness of the complex and five allotypes of ccr gene (ccrAB1, ccrAB2, ccrAB3, ccrAB4 and ccrC) in major MRSA lineages. Different combinations of these complex classes and allotypes generate various SCCmec types. Instead of the sequencing of the whole SCCmec for typing purposes, multiplex PCR schemes have been described, which amplifiy sequences specific to each mec and ccr region by SCCmec type [68, 69].

By applying the methods used for MRSA SCCmec typing in *S. pseudintermedius* isolates, two major cassette types were identified in MRSP, SCCmec types II-III and V_T in predominant clonal lineages ST 71 and ST 68 respectively [6, 49, 57] in addition to a third SCCmec type (type VII), identified in a single multidrug resistant isolate [37]

Pathogenesis of *S. pseudintermedius*

An understanding of the molecular pathogenesis of *S. pseudintermedius* disease would facilitate the design of novel therapeutics for disease prevention. *S. pseudintermedius* produces enzymes involved in virulence such as coagulase, proteases and thermonuclease. A gene similar to staphylococcal protein A (*spa*) had been characterized recently [70]. The *spa* gene encodes a cell wall protein that binds IgG from the fc portion, thereby affecting its ability to act as an opsonin [71].

S. pseudintermedius genomes contain numerous putative virulence factor genes, including genes encoding disease causing toxins such as leukocidins, exotoxins, leukotoxins, exfoliative toxin, and hemolysins in addition to super antigens and adherence factors [23, 24]. Characterization of these factors and their association with pathogenicity would be instrumental in developing more-effective strategies against *S. pseudintermedius* infections.

Virulence gene expression in *S. aureus* and host colonization are controlled by the accessory gene regulator (*agr)* locus. The *agr* locus which is known as the quorum sensing system, allows the bacterial population to act as a single organism after a certain threshold concentration of molecules is produced by the bacteria. Some bacterial species including *S. pseudintermedius* use quorum sensing as a means to optimize virulence gene expression. The relationship between *agr* groups and human *S. aureus* diseases was investigated by studying 198 *S. aureus* strains isolated from 14 asymptomatic carriers, 66 patients with suppurative infection, and 114 patients with acute toxemia [72] with 61 strains belonging to *agr* group I, 49 belonging to *agr* group II, 43 belonging to *agr* group III, and 45 belonging to group IV and it was found that *agr* group IV strains were associated with generalized exfoliative syndromes.

DNA sequence analysis of the *S. pseudintermedius agr* locus revealed an operon with five open reading frames, *agrB*, *agrD, agrC, agrA* and *hld*, encoding a classic two component regulatory system with *AgrD* encoding an auto-inducing peptide (AIP), The *S. pseudintermedius agr* system encodes an unusual AIP with a partially cyclic structure which is different from the AIP known in *S. aureus [73, 74]*. By comparing the sequence of the AIPs of *S. pseudintermedius*, it was found that the species has at least four different AIP peptides alleles [7]. The alleles were found to be shared among the members of the SIG. The inhibition of the *agr* system was proposed as a mechanism of the control of staphylococcal infections [75]; however, more research in the mechanisms of virulence is required. The therapeutic agents targeting the quorum sensing system are known as anti-pathogenic drugs and they showed some success especially for Gram-negative bacteria [76, 77]. In a recent study of quorum sensing inhibition activity of 168 plant extracts through the quantification of the delta-hemolysin toxin of a pathogenic isolate of methicillin-resistant *S. aureus* MRSA, it was found that extracts from three plants exhibited a dose-dependent response in the production of delta-hemolysin, indicating quorum sensing inhibition activity in an MRSA isolate [78]

Colonization and Immunity against Staphylococcal infections

The genus Staphylococcus which consists of at least 44 species is considered part of the natural skin flora of most mammalian species. Most of the species demonstrate host specificity and some species even demonstrate niche specificity on their particular hosts. It is known that normal flora has an important role in developing a functional mucosal immune system. There are several mechanisms that help staphylococci colonize the host skin surfaces. For example, *S. aureus* produces several surface proteins, including clumping factor B, promote adhesion to squamous cells in vitro. Clumping factor B binds to cytokeratin 10 which is a major surface protein of squamous cells [79]. *S. pseudintermedius* strains can adhere to immobilized human cytokeratin 10 suggesting that colonization of canine skin might involve similar interactions to those of *S. aureus* [80]. *spsD* and *spsL*, cell wall-associated (CWA) proteins of *S. pseudintermedius*, mediate binding to heterologous hosts. *spsD* mediated binding to cytokeratin 10. IgG specific for *spsD* and *spsL* were identified in sera from dogs with bacterial pyoderma implying that the proteins are both expressed during infection [81, 82].

Bacterial infections are the result of complex interactions between invading bacteria and host defense mechanisms. Innate immunity against Staphylococci is mainly mediated by neutrophils. The immune mechanisms underlying the host genetic predisposition to severe *S. aureus* infection in different mouse strains was studied recently [83], where mice were inoculated with *S. aureus*. The outcome of the infection was significantly different between the breeds. C57BL/6 mice were the most resistant and BALB/c mice were highly susceptible to *S. aureus* infection in terms of bacterial growth and survival. Neutrophil depleted C57BL/6 mice became completely susceptible to *S. aureus*, indicating that neutrophils are essential for resistance to infection.

The role of the humoral immune response to staphylococcal infections is not fully clear The IgG response to some staphylococcal antigens were protective in some cases [25, 84, 85]; however, a high titer of IgG against some staphylococcal antigens was not protective in other cases [26, 86]. The heterogeneity of the IgG response against staphylococcal infections most probably was affected by the genetic backgrounds of the disease causing strains and the variability of surface proteins expression. This variability should be considered in vaccine design.

The relationship between genetic background of *S. aureus* isolates, *agr* alleles in particular and disease outcome was investigated previously [72] in *S. aureus* and it was shown that a specific *agr* group was associated with generalized exfoliative syndromes. However, questions about the immune response to the different genetic backgrounds in a single species remain to be determined.

Vaccines for Staphylococci infection

Because of wide spread antibiotic resistance in the genus staphylococci, the number of effective antibiotics to treat staphylococcal infections has become limited. The search for alternative strategies to efficiently combat staphylococcal infections is urgently needed. Immunological strategies based on vaccine development or therapeutic antibodies may have significant roles in the control of staphylococcal infections [87]. Vaccines are considered one of the most promising approaches especially with the breakthrough of next generation sequencing applications which provide novel methods for selecting antigens. Most vaccine targets are directed against surface components of staphylococci [88, 89] or soluble virulence determinants such as alpha-toxin, Panton-Valentine leukocidins, or superantigenic enterotoxins [90-93].

Bioinformatics has made it possible to identify specific groups of proteins in a short time by replacing several experimental tasks using in silico prediction steps. This way of finding novel antigens has been called reverse vaccinology [94]. This method helped in the development of a vaccine for serogroup B Neisseria meningitidis. Similar studies have been made to develop a staphylococcal vaccine [89, 90, 95, 96]. They target both active and passive immunization, those including capsular polysaccharide-based vaccine [88], human immunoglobulin G (IgG) with elevated levels of antibodies to the staphylococcal fibrinogen-binding proteins *clfA* and *sdrG* [84] and exotoxoids [92]. However, a number of unresolved issues in vaccine development relating to optimal antigenic target identification, criteria for acceptable efficacy, identification of target population, optimal timing of immunization strategy, storage and cost still need to be elucidated.

For *S. pseudintermedius*, the availability of two genome sequences [23, 24] has made it easier to screen for antigenic targets especially secreted and surface exposed proteins. Future research in our laboratory involves characterization of selected *S. pseudintermedius* virulence factors and their suitability as vaccine candidates in terms of their immunogenicity and acceptable efficacy.

Concluding statement

The goal of this doctoral project is to improve the understanding of the population genetics and the immune response to *S. pseudintermedius*. The main application of MLST is studying population genetics and global epidemiology of bacterial and fungal species. We hypothesize that an expansion of the existing MLST method for *S. pseudintermedius* by the addition of four gene loci will provide a discriminatory typing scheme to identify the different genetic lineages of *S. pseudintermedius.*

The immune response to *S. pseudintermedius* is largely unexplored area of research. An understanding of *S. pseudintermedius* pathogenesis and the nature of the immune response may facilitate the development of new therapeutics for this pathogen. We hypothesize that the IgG response against *S. pseudintermedius* is necessary for the control of the infection. This response may be affected by the genetic differences between isolates and may be similar to the response to other staphylococcal species. Our first step is developing a reliable multilocus sequence typing scheme based on 8 genes and the application of the new scheme to a large number of diverse isolates. After the analysis of the genetic diversity of *S. pseudintermedius* isolates, our next step is choosing genetically diverse isolates to study the immune response using sera from patients with pyoderma and healthy controls.

Chapter 2: Multilocus Sequence Typing (MLST) for characterization of *Staphylococcus pseudintermedius*

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This chapter is a manuscript that will be submitted to an appropriate journal in summer 2012
My contributions to this paper include: (i) Gene candidates selection and primers design, (ii) PCR and sequence data analysis, (iii) the gathering and reviewing of literature, (iv) formulation of discussion topics, (v) all of the writing.

Abstract

Staphylococcus pseudintermedius has become a newly emerging methicillin-resistant pathogen. There is limited information about the population genetic structure within this species. Foundational studies of the *Staphylococcus intermedius* group examined allelic polymorphisms in four genes. In this study, four additional slowly evolving genes with allelic polymorphisms were identified from numerous candidates and were included in a multilocus sequence typing (MLST) scheme. Application of the new, eight-gene MLST method to a total of 176 diverse *S. pseudintermedius* isolates identified 106 different sequence types (STs). This represents an additional 23 sequence types beyond those identified previously. Most methicillin-resistant *S. pseudintermedius (*MRSP) isolates were further subdivided to new STs. The expanded MLST scheme should be more discriminating, give new insight into the epidemiology and the evolution of the species, and provide important information about the spread of antibiotic resistance.

Introduction

Methicillin resistance was first described in SIG in 1999 [36]. Since then the incidence of MRSP has increased dramatically in some regions [6, 20, 97]. Methicillin-resistant *S. pseudintermedius (MRSP*) are of particular concern because they typically have resistance to multiple classes of antibiotics and due to the limited number of drug choices remaining to treat serious infections caused by these organisms[12, 20, 97]. Little is known about the spread of antibiotic resistance in *S. pseudintermedius* including the rates of horizontal transmission and spread of clonal populations of resistant strains. A subspecies typing method with high discrimination should help to elucidate the origin, spatial spread of MRSP, and transmission of antibiotic resistance to susceptible strains. Mulltilocus phylogentic analysis based on four alleles has provided insight into the genetic structure of the SIG [7]. MLST was developed in 1998 [58, 59] as a general approach for typing bacteria based on the sequence variation of slowly evolving genes. MLST provides accurate, portable data useful for global epidemiology studies and studies of evolution and population biology [60-65]. MLST techniques have been developed for diverse species of bacteria and generally use at least seven alleles [58, 61, 62, 66, 67]. The development of a MLST method for *S. pseudintermedius* was undertaken to extend our knowledge of *S. pseudintermedius* population genetics and provide a typing method with discriminating power equivalent to those used for other species.

In this study an eight-gene MLST method was developed for subspecies characterization of *S. pseudintermedius.* Provisional observations suggest that methicillin resistance has been acquired by horizontal gene transfer in this species and that recipient strains have continued to evolve into new sequence types.

Materials and Methods

Bacterial strains and DNA extraction

DNA extracts from 176 previously characterized isolates of *S. pseudintermedius* from dogs (165 isolates), cats (5 isolates) and human beings (6 isolates) [6, 7, 57] were used in this study, Based on previous studies 82 were methicillin-resistant and 94 were methicillin-susceptible [6, 7, 57]. The isolates were from 11 countries. They belonged to 83 different STs based on MLST-4 [98], 9 of which included different lineages of MRSP. DNA was extracted from overnight cultures, that had been grown at 37°C on blood agar plates, using either commercial DNA extraction Kits [7, 57] or glass bead extraction. For the latter, bacteria derived from a single colony were suspended in 0.5 ml of 1M Tris EDTA buffer pH 7.5, mixed with an equal volume of glass beads and subjected to pulsed vortexing for 5 min for cell disruption and DNA release. Supernatant of this mixture was stored at -20° C and used as template in PCR assays.

Selection and screening of genes for potential use in MLST

Sequences encoding open reading frames were identified from contiguous sequences obtained using next generation sequencing technology (Vanderbilt University, Genome Technology Core (Illumina technology) and the University of Florida Interdisciplinary Center for Biotechnology Research (Roche 454 technology) with genomic DNA isolated from *S. pseudintermedius* isolate 06.3228 (canine isolate from Tennessee, USA). *Staphylococcus aureus* genome sequences available in GenBank (accession number CP000255, CP000732, CP000253, CP000730, AP009324, AP009351, CP000736 and CP000045) were aligned using the BLASTN algorithm [99] with the National Center for Biotechnology Information (NCBI) online resource and used as a reference to predict which *S. pseudintermedius* genes were most likely to have polymorphic sites. Candidate genes were chosen for testing using PCR primers designed with the Primer 3 plus program [100] and obtained commercially (Integrated DNA Technologies). A panel of 15 *S. pseudintermedius* isolates with different genetic backgrounds was used for allele selection. PCR products, when obtained from all isolates, were sequenced and MLST targets were selected based upon the greatest number of alleles. These included loci

contained within adenylosuccinate synthetase (*purA*), formate dehydrogenase (*fdh*), perfringolysin O regulatory protein (*pfoR*) and sodium: sulfate symporter (*sar*) genes (Table 1).

PCR amplification of gene fragments and sequencing

A thermal cycling protocol for PCR amplification of 8 loci including *cpn 60, tuf, pta* and *agrD* was developed. The PCR reaction consisted of initial denaturation at 95°C for 90 seconds, followed by 35 cycles of annealing for 30 seconds at 52°C, extension for 1 min at 72°C, denaturation for 30 seconds at 94°C, then annealing at 52°C for 30 seconds and a final extension at 72°C for 5 min. A 2x mastermix containing rTaq polymerase (Takara, USA) was used to establish the optimal amplification conditions.

PCR products were resolved and visualized by electrophoresis in gels containing 1.4% agarose and 0.5 µg/ml ethidium bromide. Products of expected sizes were treated to destroy single-stranded DNA (ExoSap-IT, USB Corp., Cleveland, OH) and sequenced at The University of Tennessee Molecular Biology Resource Facility (Knoxville, TN) or Baseclear (Leiden, the Netherlands).

DNA Sequence Analysis

Sequences were analyzed using the commercial software packages Lasergene (DNAStar, Wisconsin, USA) and Bionumerics vs. 6.1 (Applied Maths, Belgium). The loci in the extended panel, presented relative to reference genes (and their GenBank accession numbers), were: *purA*: 568 bp – 968 bp (JF275101), *fdh*: 888bp - 1236 bp (JF275100), *pfoR:* 248 bp – 641 bp (JF275102), and *sar:* 246 bp – 683 bp (JF275103). Table 2.5A showed the trimming sequence for the 4 genes. The MLST data set was used to construct a minimum spanning tree (MST) using Bionumerics software. A single, unique, ST was used to represent each data point.

Results

Four of forty-five candidate genes fulfilled criteria for inclusion in a MLST scheme and produced the greatest number of alleles. These included adenylosuccinate synthetase gene *purA*, formate dehydrogenase gene *fdh*, sodium: sulfate symporter gene *sar* and perfringolysin O regulatory gene *pfoR*. The *purA* gene sequences had 11 polymorphic sites and 20 different alleles were detected among the tested isolates (Figure 1 and

table 2.1A). All predicted amino acid sequences were identical except for two amino acid substitutions in allele 11 and one amino acid substitution in allele 15. Five polymorphic sites were detected in *fdh*, all encoding the same amino acids. The polymorphisms yielded 5 different alleles, three of which occurred in MRSP. *fdh* allele 1 predominated and was present in both susceptible and resistant isolates (Figure 2 and table 2.2A). For the pfoR gene 10 polymorphic sites and 14 different alleles were identified. Seven pfoR alleles occurred in both MRSP and MSSP isolates (Figure 4 and table 2.3A). All encoded amino acids were the same except allele 6 and allele 11 which contained stop codons. Eight polymorphic sites were detected in the *sar* gene resulting in 8 alleles (Figure 3 and table 2.4A). All were silent substitutions except one that creates an alanine to threonine amino acid change in the deduced amino acid sequence. MRSP isolates shared only three *sar* alleles (Table 3 and figure 4), and alleles 1 and 2 were highly represented among both susceptible and resistant isolates.

Using the eight gene MLST, 106 STs were detected. Allelic polymorphisms were identified for all seven MRSP tested for which more than one sample was available except ST 71 and MRSP were contained within each major phylogenetic lineage for each allele (Figures 1-4).

With the expanded MLST scheme 3 feline isolates had unique STs. ST 5 was previously identified from both canine and feline samples, however, the expanded MLST method identified six differences out of eight loci between isolates from the two hosts. Likewise, ST 19, the one non-unique human ST, differed at three loci compared to its ST 19 canine counterpart when the expanded method was applied. All six samples included in the study from Japan had unique STs with the 8 loci scheme including 2 that were newly discriminated. Isolates that had identical STs among previously determined United States STs 15, 17, 19, 26, 39, and 61 but were from different geographic origins, were distinguished when the 8 loci typing scheme was used. It did not discriminate between ST 71s from feline and canine origin or from various geographic regions.
MST analysis (Figure 5) formed four major branches radiating from ST 26 with methicillin resistant STs on three of the branches. Isolates from the United States and Europe were present on all branches. There was no apparent clustering of STs from different host species.

Table 2.1: Primers used for PCR amplification

Gene locus	Primer 5'- 3'	position	Primer Sequence	product size
Adenylosuccinate synthetase *purA*	*purA* Forward	505 bp	GATTACTTCCAAGGTATGTTT	490 bp
	purA Reverse	995 bp	TCGATAGAGTTAATAGATAAGTC	
Formate dehydrogenase *fdh*	*fdh* Forward	878 bp	TGCGATAACAGGATGTGCTT	408 bp
	fdh Reverse	1286 bp	CTTCTCATGATTCACCGGC	
Perfringolysin O regulatory protein *pfoR*	*pfoR* Forward	193 bp	ATCGTCGCTTTACGGTCACCT	447 bp
	pfoR Reverse	678 bp	GCTGCAGCGGGTCTGACG	
Sodium: sulfate symporter *sar*	*sar* Forward	184 bp	GGATTTAGTCCAGTTCAAAATTT	521 bp
	sar Reverse	705 bp	GAACCATTCGCCCCATGAA	

Table 2.2: The allelic profiles of MRSP sequence types

ST based on MLST-4	*purA* alleles	*fdh* alleles	*pfoR* alleles	*sar* alleles	Number of strains tested	ST based on MLST-8
5	1, 16	2	1, 5	2	2	5, 5.5
26	5, 13	2	2, 3	1, 5	7	26, 26.1, 26.2, 26.3
29	2, 17	2, 1	1, 2, 4	1, 2	7	29, 29.1, 29.2
68	1,5	1,2	1, 5	1	46	68, 68.1, 68.2
69	1	3	5	1	1	69
71	1	1	6	2	18	71
100	1, 4	2, 3	5, 10	1	2	100.1, 100.2
105	2	2	2	1	1	105
118	5	2	2	2	2	118

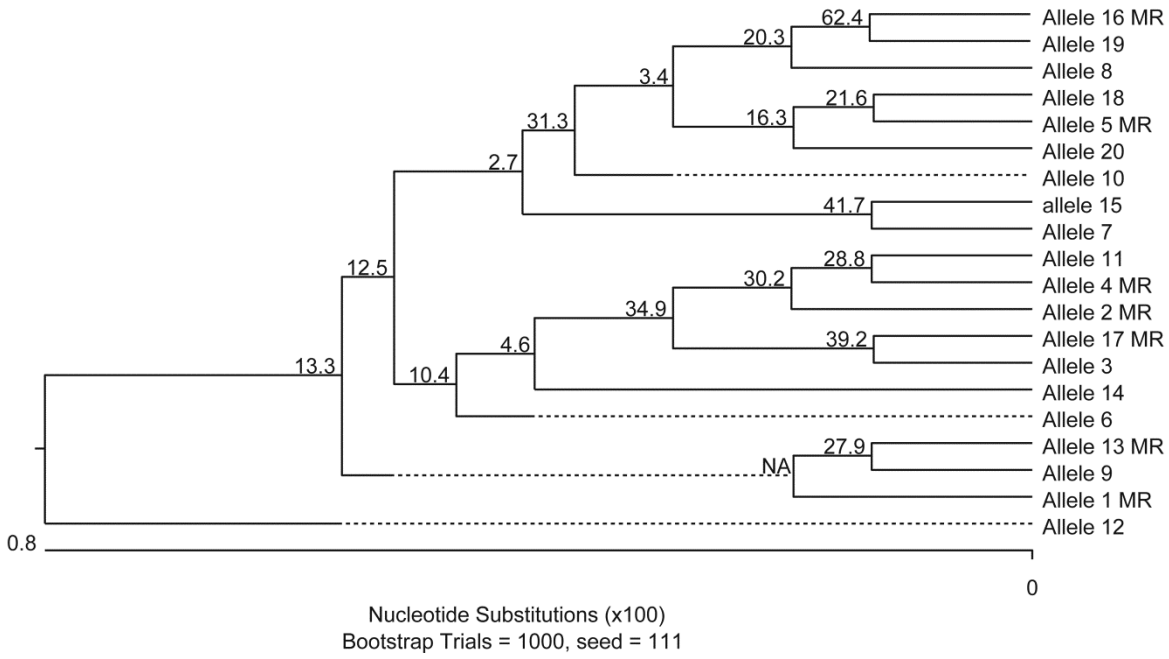

Figure 2.1. Phylogenetic tree of *purA* alleles constructed with the megAlign function of the software packages Lasergene (DNAStar, Wisconsin, USA) based on the sequence data of the *purA* gene. One thousand bootstrap replicates were used to determine the confidence in the tree.

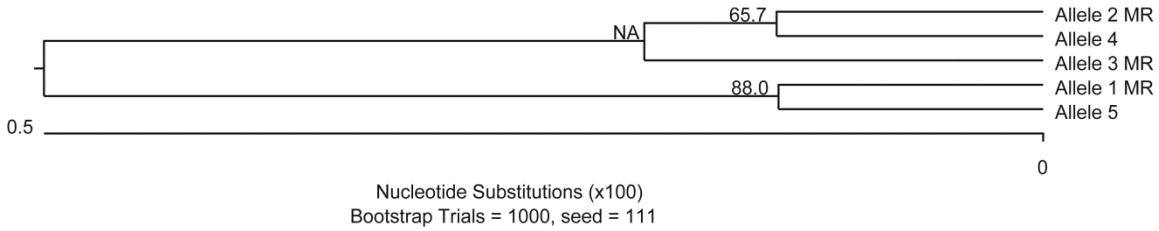

Figure 2.2. Phylogenetic tree of *fdh* alleles constructed with the megAlign function of the software packages Lasergene (DNAStar, Wisconsin, USA) based on the sequence data of the *fdh* gene. Methicillin resistant alleles MR distribute in each lineage. One thousand bootstrap replicates were used to determine the confidence in the tree.

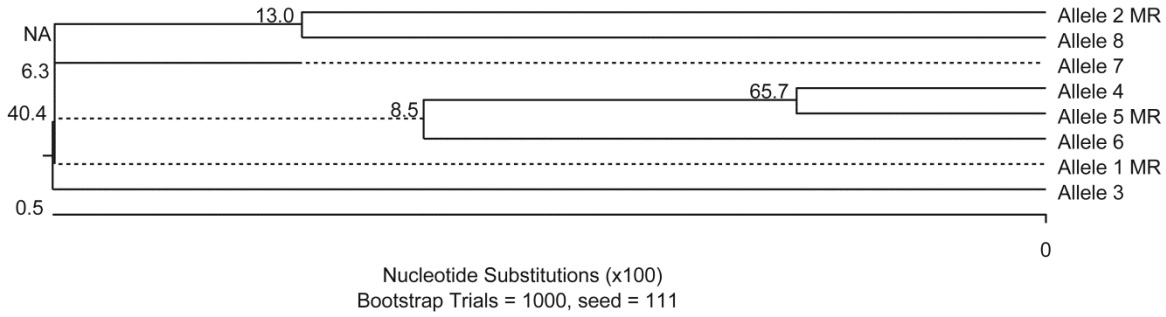

Figure 2.3. Phylogenetic tree based on the sequence data of the *sar* gene showing 3 methicillin resistant alleles out of 8 alleles. One thousand bootstrap replicates were used to determine the confidence in the tree.

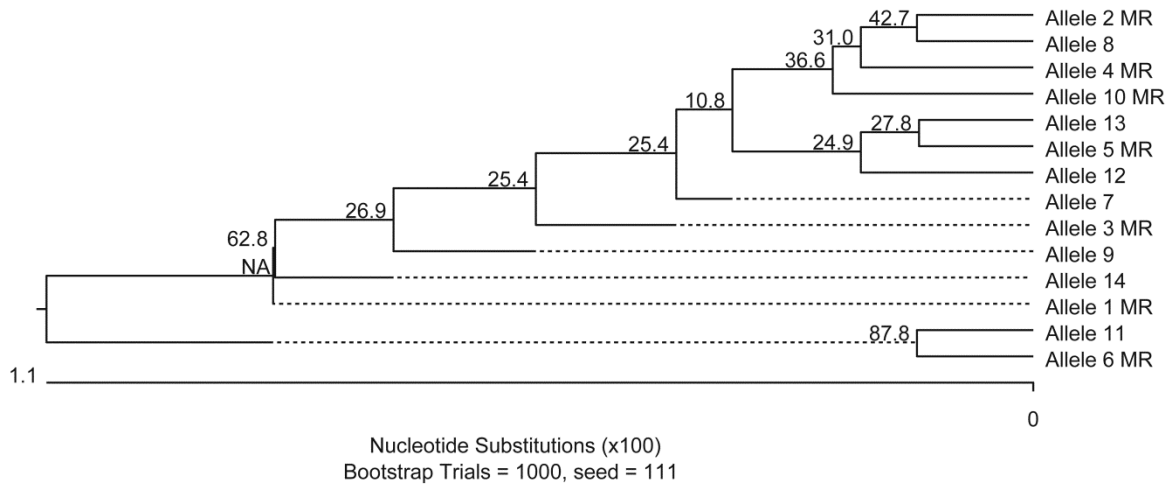

Figure 2.4. Phylogenetic tree based on the sequence data of the *pfoR* gene showing 14 alleles and the methicillin-resistant alleles represented in each lineage. Eighty seven bootstrap value between allele 11 and allele 6 indicates the relatedness between them.

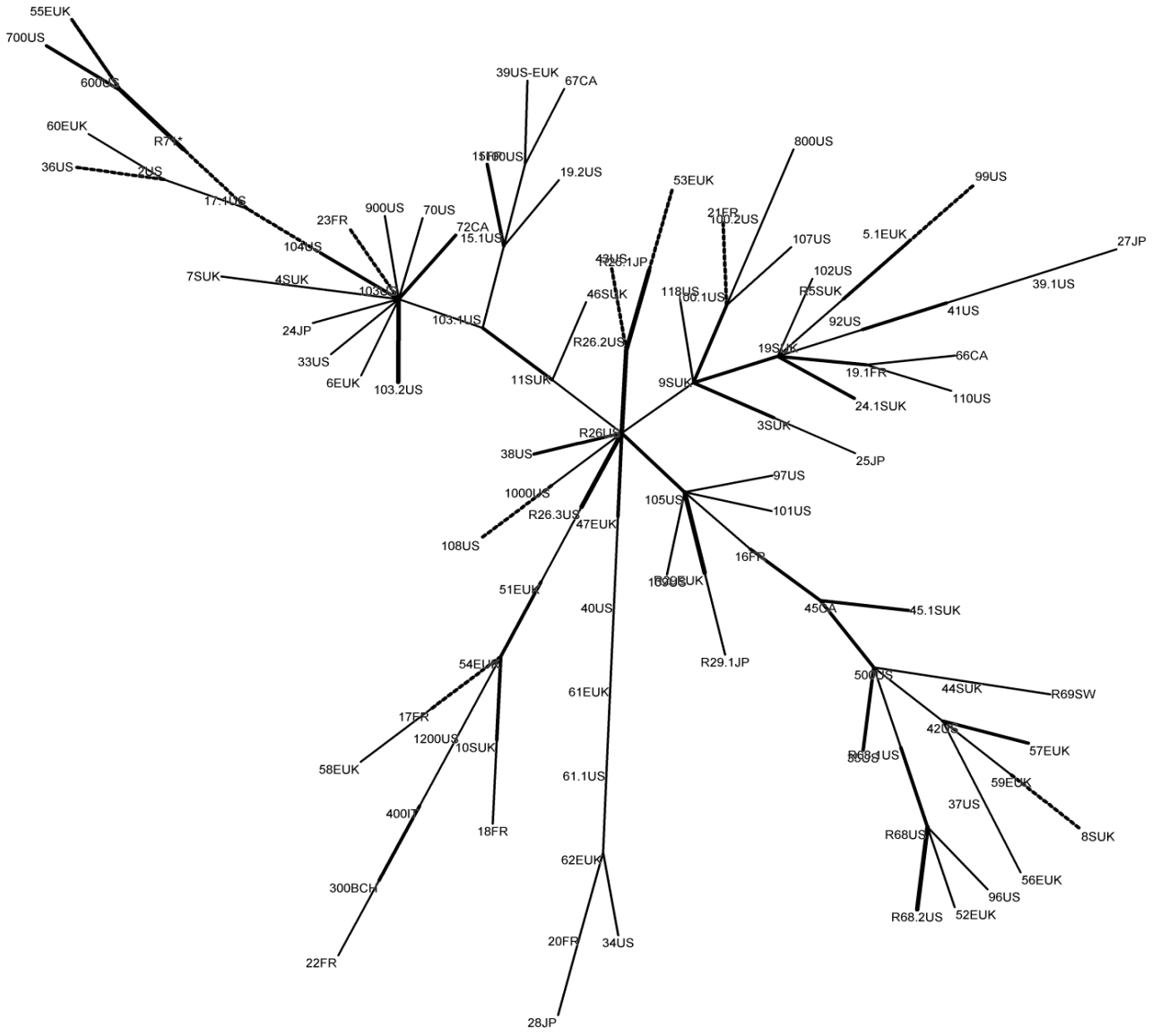

Figure 2.5. Minimum spanning tree of MLST data. Sequence types are represented by numbers. Methicillin-resistant isolates have an “R” prefix and the country of origin is represented with an extension of US (United States), IT (Italy), BCH (Switzerland), SUK (Scotland), EUK (England), FR (France), JP (Japan), CA (Canada), or SW (Sweden). ST 71 was isolated from multiple locations including Germany, Sweden, The United States, Italy, Switzerland, and The Netherlands. Single locus variant STs are connected by a thick line, double locus variants by an intermediate line, triple locus variants by a thin line, variants that differ by four loci with a dashed line, and STs that vary by more than 4 loci by a dotted line.

Table 2.3: Distribution of *Staphylococcus pseudintermedius* sequence types using MLST-8 with isolates from different hosts and geographic origins.

					MLST-8							
					MLST-4 (Bannoehr et al. 2007)			***New alleles(this study)***				
	Strain	Host	Geographical Origin	ST	*tuf*	*cpn60*	*pta*	*agrD*	*purA*	*Fdh*	*pfoR*	*sar*
1	ED 99	Canine	Scot. UK	2	1	9	1	1	1	2	4	1
2	95-062295	Canine	USA, TN	2.1	1	9	1	1	8	3	14	4
3	M 1351/03	Canine	Scot.UK	3	2	7	18	2	1	2	3	1
4	M407/03	Canine	Scot.UK	4	1	11	1	3	8	2	3	1
5	M 1332/03	Canine	Scot.UK	5	1	2	1	2	1	2	1	2
6	3414	Feline	Eng.UK	5.1	1	2	1	2	16	2	5	2
7	M 695/99	Canine	Scot, UK	6	2	9	1	1	8	4	12	1
8	M 741/99	Canine	Scot.UK	7	1	13	1	3	8	2	13	6
9	M 1337/03	Canine	Scot.UK	8	1	21	2	2	7	3	1	4
10	M1333/03	Canine	Scot.UK	9	2	7	1	2	1	2	2	1
11	M707/99	Canine	Scot.UK	10	2	2	4	1	7	2	3	1
12	M 721/99	Canine	Scot.UK	11	2	3	1	3	4	2	2	1
13	HT20030683	Canine	France	15	2	6	1	2	15	1	5	1
14	08.1988b	Canine	USA, TN	15.1	2	6	1	2	4	1	4	1
15	HT20030684	Canine	France	16	2	7	8	1	14	2	2	1
16	HT20030685	Canine	France	17	2	6	4	1	4	4	1	1
17	6.3096	Canine	USA, TN	17.1	2	6	4	1	8	3	7	1
18	HT20030686	Canine	France	18	2	2	4	2	13	2	5	1
19	M 449/ 06	Canine	Scot.UK	19	2	8	1	2	1	2	1	1
20	N 900260	Human	France	19.1	2	8	1	2	1	2	3	2
21	08.1752a	Canine	USA, TN	19.2	2	8	1	2	4	1	8	2
22	N 910/201	Human	France	20	1	2	1	1	4	4	3	2
23	N940276	Human	France	21	2	7	2	3	20	2	3	8
24	N940453	Human	France	22	2	7	4	2	7	4	5	2
25	LY19990344	Human	France	23	9	6	1	3	8	4	3	7
26	AV 8001	Canine	Japan	24	2	9	1	2	13	1	3	5
27	M575/06	Canine	Scot.UK	24.1	2	9	1	2	1	4	1	1
28	AV 8002	Canine	Japan	25	2	7	2	2	4	3	3	1
29	NA 4	Canine	USA, TX	26	2	10	1	4	5	2	2	1
30	NA 45	Canine	USA, WI	26	2	10	1	4	5	2	2	1
31	NA 6	Canine	USA, TX	26	2	10	1	4	5	2	2	1

Table 2.3. Continued

				MLST-8								
					MLST-4		***New alleles(this study)***					
	Strain	Host	Geographical Origin	ST	*tuf*	*cpn60*	*pta*	*agrD*	*purA*	*Fdh*	*pfoR*	*sar*
32	NA 49	Canine	USA, WI	26	2	10	1	4	5	2	2	1
33	AV 8012	Canine	Japan	26.1	2	10	1	4	13	2	2	5
34	NA 3	Canine	USA, TX	26.2	2	10	1	4	5	2	2	5
35	NA 43	Canine	USA, WI	26.3	2	10	1	4	5	2	3	1
36	AV 8010	Canine	Japan	27	1	11	1	2	8	1	3	4
37	AV 8024	Canine	Japan	28	2	7	1	1	13	4	3	2
38	3279	Canine	Eng. UK	29	2	2	1	1	2	2	2	1
39	2.09E+11	Canine	Netherland	29	2	2	1	1	2	2	2	1
40	V08016361	Canine	Netherland	29	2	2	1	1	2	2	2	1
41	2.09E+09	Canine	Netherland	29	2	2	1	1	2	2	2	1
42	AV 8033	Canine	Japan	29.1	2	2	1	1	17	2	1	2
43	2.08E+11	Canine	Netherland	29.2	2	2	1	1	2	2	4	2
44	96022396	Feline	USA	33	2	2	1	3	13	1	2	1
45	93071493	Canine	USA	34	1	13	11	1	14	2	3	2
46	94060294	Canine	USA	35	1	2	12	3	19	2	2	1
47	94-062394	Canine	USA	36	1	9	1	4	7	1	9	4
48	94072/594	Canine	USA	37	1	2	2	1	4	2	1	3
49	95011195	Canine	USA	38	5	13	11	4	7	2	4	1
50	95062/195	Canine	USA	39	1	7	1	2	5	4	4	1
51	8193	Canine	Eng.UK	39	1	7	1	2	5	4	4	1
52	95072195	Canine	USA	39.1	1	7	1	2	5	2	3	4
53	96022/296	Canine	USA	40	1	7	1	1	5	2	1	1
54	96 030 796	Canine	USA	41	1	13	1	2	4	2	3	1
55	96-032996	Canine	USA	42	1	2	2	3	4	2	4	1
56	96041096	Canine	USA	43	1	14	1	4	3	5	2	5
57	M 543/ 06	Canine	Scot.UK	44	1	2	1	3	1	3	8	1
58	BH 47	Canine	Canada	45	1	7	8	3	14	2	2	1
59	M629/06	Canine	Scot.UK	45.1	1	7	8	3	1	4	2	1
60	M657/06	Canine	Scot.UK	46	2	15	4	3	8	2	2	1
61	8016	Canine	Eng.UK	47	1	16	1	4	5	2	2	1
62	8185	Canine	Eng. UK	51	2	8	4	4	1	2	3	1
63	388	Canine	Eng. UK	52	2	18	4	3	2	1	1	1
64	9075	Canine	Eng.UK	53	2	19	4	4	13	4	2	4
65	9162	Canine	Eng. UK	54	2	9	4	1	1	2	3	1
66	690	Feline	Eng.UK	55	1	8	5	4	3	1	3	2

Table 2.3. Continued

				MLST-8								
					MLST-4			***New alleles(this study)***				
	Strain	Host	Geographical Origin	ST	*tuf*	*cpn60*	*pta*	*agrD*	*purA*	*Fdh*	*pfoR*	*sar*
67	8239	Canine	Eng.UK	56	1	2	7	1	3	2	1	1
68	8478	Canine	Eng.UK	57	1	2	2	4	4	2	8	1
69	3708	Canine	Eng, UK	58	2	9	2	1	4	4	1	5
70	129Ab	Canine	Netherland	58.1	2	9	2	1	2	6	-5	1
71	4229	Canine	Eng.UK	59	1	2	2	3	18	3	1	1
72	2431	Canine	Eng.UK	60	1	9	3	1	3	3	4	4
73	9318	Canine	Eng.UK	61	1	20	1	1	3	2	5	1
74	8.153	Canine	USA, TN	61.1	1	20	1	1	7	1	3	1
75	8639	Canine	Eng.UK	62	1	3	1	1	7	2	3	2
76	BH 04	Canine	Canada	66	1	7	4	2	1	2	3	2
77	BH 34	Feline	Canada	67	1	27	2	2	3	1	2	1
78	Can 5	Canine	USA,	68	1	10	4	3	1	1	1	1
79	M06-13	Canine	USA	68	1	10	4	3	1	1	1	1
80	6.1041	Canine	USA, TN	68	1	10	4	3	1	1	1	1
81	6.1065	Canine	USA, TN	68	1	10	4	3	1	1	1	1
82	6.1164	Canine	USA, TN	68	1	10	4	3	1	1	1	1
83	6.14	Canine	USA, TN	68	1	10	4	3	1	1	1	1
84	6.146	Canine	USA, TN	68	1	10	4	3	1	1	1	1
85	6.255	Canine	USA, TN	68	1	10	4	3	1	1	1	1
86	6.2584	Canine	USA, TN	68	1	10	4	3	1	1	1	1
87	6.3187	Canine	USA, TN	68	1	10	4	3	1	1	1	1
88	06.4364a	Canine	USA, TN	68	1	10	4	3	1	1	1	1
89	06.4899e	Canine	USA, TN	68	1	10	4	3	1	1	1	1
90	6.632	Canine	USA, TN	68	1	10	4	3	1	1	1	1
91	6.815	Canine	USA, TN	68	1	10	4	3	1	1	1	1
92	6.3228	Canine	USA, TN	68	1	10	4	3	1	1	1	1
93	07.1605a	Canine	USA, TN	68	1	10	4	3	1	1	1	1
94	7.297	Canine	USA, TN	68	1	10	4	3	1	1	1	1
95	07.372a	Canine	USA, TN	68	1	10	4	3	1	1	1	1
96	07.425a	Canine	USA, TN	68	1	10	4	3	1	1	1	1
97	7.5662	Canine	USA, TN	68	1	10	4	3	1	1	1	1
98	7.5781	Canine	USA, TN	68	1	10	4	3	1	1	1	1
99	7.5987	Canine	USA, TN	68	1	10	4	3	1	1	1	1
100	07.603a	Canine	USA, TN	68	1	10	4	3	1	1	1	1
101	07.617a	Canine	USA, TN	68	1	10	4	3	1	1	1	1

Table 2.3. Continued

				MLST-8								
				MLST-4			***New alleles(this study)***					
	Strain	Host	Geographical Origin	ST	*tuf*	*cpn60*	*pta*	*agrD*	*purA*	*Fdh*	*pfoR*	*sar*
102	7.6388	Canine	USA, TN	68	1	10	4	3	1	1	1	1
103	7.547	Canine	USA, TN	68	1	10	4	3	1	1	1	1
104	8.128	Canine	USA, TN	68	1	10	4	3	1	1	1	1
105	8.22	Canine	USA, TN	68	1	10	4	3	1	1	1	1
106	8.324	Canine	USA, TN	68	1	10	4	3	1	1	1	1
107	8.355	Canine	USA, TN	68	1	10	4	3	1	1	1	1
108	8.36	Canine	USA, TN	68	1	10	4	3	1	1	1	1
109	08.39a	Canine	USA, TN	68	1	10	4	3	1	1	1	1
110	8.494	Canine	USA, TN	68	1	10	4	3	1	1	1	1
111	8.511	Canine	USA, TN	68	1	10	4	3	1	1	1	1
112	08.521a	Canine	USA, TN	68	1	10	4	3	1	1	1	1
113	8.1043	Canine	USA, TN	68	1	10	4	3	1	1	1	1
114	8.1294	Canine	USA, TN	68	1	10	4	3	1	1	1	1
115	NA 37	Canine	USA, NC	68	1	10	4	3	1	1	1	1
116	NA 64	Canine	USA, CA	68	1	10	4	3	1	1	1	1
117	NA 50	Canine	USA, WI	68	1	10	4	3	1	1	1	1
118	NA 10	Canine	USA, TX	68	1	10	4	3	1	1	1	1
119	NA 27	Canine	USA, WA	68	1	10	4	3	1	1	1	1
120	NA 24	Canine	USA, NC	68	1	10	4	3	1	1	1	1
121	Sp80	Canine	Netherland	68	1	10	4	3	1	1	1	1
122	NA 127	Canine	USA, GA	68	1	10	4	3	1	1	1	1
123	NA 133	Canine	USA, NJ	68	1	10	4	3	1	1	1	1
124	07.676 C	Canine	USA, TN	68.1	1	10	4	3	1	2	5	1
125	08.547a	Canine	USA, TN	68.2	1	10	4	3	5	1	1	1
126	13	Canine	Sweden	69	1	25	1	1	1	3	5	1
127	Can 10	Canine	USA	70	1	7	1	3	13	1	3	1
128	HH4	Feline	Germany	71	1	9	2	1	1	1	6	2
129	7577-06	Canine	Sweden	71	1	9	2	1	1	1	6	2
130	Gil 1	Canine	Germany	71	1	9	2	1	1	1	6	2
131	BD19698-06	Canine	Sweden	71	1	9	2	1	1	1	6	2
132	HH1	Canine	Germany	71	1	9	2	1	1	1	6	2
133	8.1661	Canine	USA, TN	71	1	9	2	1	1	1	6	2
134	08.3174d	Canine	USA, TN	71	1	9	2	1	1	1	6	2
135	E 34	Canine	Italy	71	1	9	2	1	1	1	6	2
136	E 36	Canine	Italy	71	1	9	2	1	1	1	6	2
137	NA 18	Canine	USA, CA	71	1	9	2	1	1	1	6	2

Table 2.3. Continued

					MLST-8							
					MLST-4			***New alleles(this study)***				
	Strain	Host	Geographical Origin	ST	*tuf*	*cpn60*	*pta*	*agrD*	*purA*	*Fdh*	*pfoR*	*sar*
138	E 42	Canine	Italy	71	1	9	2	1	1	1	6	2
139	KM1381	Canine	Switzerland	71	1	9	2	1	1	1	6	2
140	IMD07824	Canine	Switzerland	71	1	9	2	1	1	1	6	2
141	NA 126	Canine	USA, FL	71	1	9	2	1	1	1	6	2
142	BH 01	Canine	Canada	72	2	26	1	3	8	1	1	1
143	KM241	Canine	Switzerland	73	2	13	19	2	1	1	3	2
144	8.2211	Canine	USA, TN	92	1	8	1	2	6	2	3	1
145	06.1705a	Canine	USA, TN	96	1	32	23	3	1	1	3	1
146	07.367C	Canine	USA, TN	97	1	8	1	1	7	2	2	1
147	8.1781	Canine	USA, TN	99	1	27	8	2	5	3	5	2
148	7.1447	Canine	USA, TN	100	2	7	1	3	1	2	5	1
149	NA 31	Canine	USA, WA	100	2	7	1	3	4	3	10	1
150	07.182a	Canine	USA, TN	101	2	8	1	1	4	2	3	1
151	8.1721	Canine	USA, TN	102	2	8	2	2	6	2	2	1
152	06.2735a	Canine	USA, TN	103	2	9	1	3	8	1	3	1
153	07.188a	Canine	USA, TN	103	2	9	1	3	4	2	4	1
154	8.1791	Canine	USA, TN	103	2	9	1	3	8	1	3	2
155	07.169a	Canine	USA, TN	104	2	9	2	3	8	1	1	1
156	7.5066	Canine	USA, TN	105	2	10	1	1	2	2	2	1
157	2.09E+09	Canine	Netherland	106	2	13	1	2	4	4	3	2
158	2.081E+09	Canine	Netherland	106	2	13	1	2	4	4	3	2
159	V0708754	Canine	Netherland	106	2	13	1	2	4	4	3	1
160	06.2381a	Canine	USA, TN	107	2	13	1	3	10	2	9	1
161	07.281a	Canine	USA, TN	108	2	13	1	4	9	3	1	3
162	06.3998a	Canine	USA, TN	109	2	27	1	1	8	2	1	1
163	07.257a	Canine	USA, TN	110	2	27	1	2	3	2	3	4
164	V0805157	Canine	Netherland	111	2	7	1	4	4	4	3	2
165	V0709390-3	Canine	Netherland	114	1	7	4	3	4	1	3	1
166	NA 11	Canine	USA, TX	118	2	7	11	2	5	2	2	2
167	NA 12	Canine	USA, TX	118	2	7	11	2	5	2	2	2
168	NA 67	Canine	USA, CA	159	1	2	8	3	1	2	2	1
169	NA 55	Canine	USA, CA	71	1	9	2	1	1	1	6	2
170	NA 23	Canine	Canada	71	1	9	2	1	1	1	6	2
171	NA 47	Canine	USA, WI	800	New	7	1	1	4	3	3	1
172	NA 20	Canine	Canada	900	2	New	1	2	1	1	3	1
173	NA 85	Canine	USA, IN	158	2	9	1	4	3	2	1	1

Table 2.3. Continued

	Strain	Host	Geographical Origin	ST	MLST-8							
					MLST-4		***New alleles (this study)***					
					tuf	*cpn60*	*pta*	*agrD*	*purA*	*Fdh*	*pfoR*	*sar*
174	8.1748	Canine	USA, TN	157	1	27	1	2	11	1	4	1
175	08.3174a	Canine	USA, TN	156	2	13	4	1	3	1	3	1
176	NA 128	Canine	USA, NY	20.1	1	2	1	1	4	2	2	1

Discussion

The origin of methicillin resistance among *S. pseudintermedius* is unknown. The geographical concentration of two clonal populations in Europe and North America, ST 71 and ST 68 suggest recent acquisition of the *mecA* gene. [6, 7, 57]. These observations were dependent on studies with restricted interpretation because only four alleles were used for genetic analysis and the geographic areas sampled were limited. In the current study five of the nine MRSP lineages were further divided with the use of an expanded panel of alleles. Since a low number of MRSP were used to establish the MLST scheme it is likely that many of the MRSP clones will be further divided when this method is applied in population studies.

The highest degree of polymorphism among the four genes studied occurred in the *purA* gene locus and it seems to contain four distinct phylogenetic lineages. *purA* allele 1 was present in a majority of the MRSP tested and may have a selective advantage, however, almost all predicted *purA* products had the same amino acid sequences as the one encoded by allele 1. The house keeping functions of *purA, fdh*, and *sar* gene products may be essential for bacterial survival and thus there are likely restrictions on their amino acid composition. The *fdh* gene encodes proteins involved in the oxidation of formate to bicarbonate and all the mutations in *fdh* were silent. *sar* gene products encode proteins involved in substrate uptake and the accumulation of small solutes within the cell. The polymorphisms in *sar* were silent except for one sense mutation. *pfoR* gene is similar in sequence to a gene encodes a protein involves in the regulation of the perfringolysin 0 (theta-toxin) gene in *Clostridium perfringens*. The function of *pfoR* in *S. pseudintermedius* is unclear. Most of the *pfoR* polymorphisms were silent except for one that caused premature termination of the protein by the formation of a stop codon at amino acid 131 in the deduced protein. The alleles used for MLST belong to genes that are intrinsic to *S. pseudintermedius*. Such housekeeping genes with high polymorphism represent ideal and reliable alleles for MLST of *S. pseudintermedius* to be used in combination to previously used 4 alleles[98].

The proposed MLST scheme identified 106 unique sequence types, 23 more than had been previously recognized. Thus, it has improved discriminatory ability compared to the use of four alleles.

The origin of emerging strains is often studied by the use of eBURST analysis [101]. This method of analyzing MLST data has been used for the identification of founder strains and to obtain an overview of population dynamics. Due to the high diversity of the isolates used in this study and the large number of alleles and connecting a large number of strains. Data from a broader sampling of strains will be required to make this comparison with the higher stringency scheme.

MST analysis suggests ST 26 is a founder at the center of each radiating branch. The numbers of ST 26 isolates characterized in this study were small and all were methicillin-resistant. It is unlikely that methicillin resistance arose prior to the expansion of *S. pseudintermedius* as acquisition of resistance is a fairly recent event. Therefore, it is possible that a methicillin-susceptible precursor ST 26 isolate served as a founder and that methicillin resistance was acquired more recently in this ST by lateral transfer. Frequency of ST occurrence was not included in the analysis because an appropriate dataset that reflects unbiased sampling of a broad population is not currently available.

In this study, we report the development of an 8 loci MLST scheme for S. *pseudintermedius*. We propose that data generated by this method can be globally shared to obtain clearer picture of the evolution of methicillin resistance among *S. pseudintermedius* and improve our understanding of the population genetics of this species. The new ST identifiers used for the expanded MLST scheme are intended only to illustrate relationships within this study. Vincent Perreten(vincent.perreten@vbi.unibe.ch) serves as curator for assignment of STs for the *S. pseudintermedius* research community and has generously agreed to continue to do so for the expanded MLST system.

Chapter 3: Immune Response to *Staphylococcus pseudintermedius*

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My contributions to this paper include: (i) experimental design, isolates selection and antigen preparation, (ii) flow cytometry analysis and western blot, (iv) the gathering and reviewing of literature, (iv) formulation of discussion topics, (v) all of the writing except the clinical portion.

Abstract

Little is known about the immune response to *S. pseudintermedius*, the important canine pathogen that has developed a high prevalence of methicillin resistance. Understanding the nature of the protective immune response would facilitate the design of new therapeutics. The aim of this study was to characterize the nature of the immunoglobulin G (IgG) response in 14 dogs with pyoderma and 14 healthy dogs with no history of pyoderma to antigens from 5 genetically distinct *S. pseudintermedius* isolates and 5 different staphylococcal species. Serum samples were obtained at the time of patient admission and 3 to 8 weeks later. The staphylococcal proteins were analyzed using three techniques; western blot, flow cytometry and Enzyme-Linked ImmunoSorbant Assay (ELISA). The results indicated significant differences in the protein banding pattern between the five different *S. pseudintermedius* isolates as revealed by western blot. All 28 dogs showed IgG reactivity to multiple *S. pseudintermedius* antigens. Antibodies to some antigens were seen only in samples from infected dogs. The IgG response

was correlated in part with the outcome of infection, suggesting the potential protective nature of this response. Some variations were shown in the reactivity of the five isolates with the dog sera in both groups using flow cytometry which indicated a significant difference in the surface exposed antigens between different genetic backgrounds. The highest reactivity was to a methicillin-susceptible clinical isolate of ST96 and to the isolate of ST 71 that represents a methicillin-resistant clone widely distributed in Europe. The result was confirmed with ELISA. Among the other staphylococcal species tested, *S. delphini* had least reactivity with the canine sera.

Introduction

A major challenge for healthcare in the Twenty-first century is increasing levels of resistance to antimicrobial compounds by some pathogenic bacteria. Multi-drug resistance emerged recently as a problem in the important canine bacterium *S. pseudintermedius* [6, 57, 98, 102]. Considering that treatment options in veterinary medicine are limited, an effective vaccine to prevent infections is urgently needed.

Work with the human pathogen *S. aureus* has demonstrated the potential challenges of developing a vaccine for staphylococci. *S. aureus* is equipped with multiple virulence and immune escape factors, in addition to the genetic heterogeneity of the species. Two *S. aureus* strains can differ by up to 20% of their genome, even when they share the core genome. The variable genome consists of mobile genetic elements (MGE), such as pathogenicity islands and phages, which encode numerous secreted virulence factors, including toxins, exoenzymes and immune modulators [103-105]. In addition to genome diversity, there are remarkable strain differences in the regulation of gene expression resulting in pronounced heterogeneity of *S. aureus* at the protein level. Protein variability is particularly obvious in the extracellular and cell surface proteomes, which directly contact the host immune system [106, 107]. From the two available *S. pseudintermedius* genomes [23, 24] there are numerous putative virulence factors; however the association between these factors and pathogenesis is unclear. A clearer understanding of the features of the host immune response that is necessary for the generation of protective immunity is needed.

The innate immune response to staphylococci is mediated mainly by polymorphonuclear leukocytes (PMNs or neutrophils). Phagocytosis of bacteria by neutrophils triggers production of reactive oxygen species (ROS) and release

of microbiocidal granule components into phagosomes. Although neutrophils are effective, some bacteria like *S. aureus* resist their bactericidal effect by changing gene expression to enhance survival of the bacterium. Voyich et al., studied *S. aureus*-PMN interactions using strains isolated from community acquired (CA) and hospital acquired (HA) infections and found that genes involved in capsule synthesis, gene regulation, oxidative stress, and virulence, were up-regulated following ingestion of the pathogen. Phagocytosis of strains from CA infections induced changes in gene expression not observed in other strains [108].

Immunoglobulin G (IgG) plays an essential role in the humoral immune system for the control of bacterial infections [109]. One of the important functions of this antibody is opsonizing bacteria and improving their recognition by phagocytic leukocytes [110]. IgG can also activate the classical complement pathway leading to additional opsonization by the complement C3b/iC3b fragments, the formation of membrane attack complex and the generation of anaphylatoxins [109, 111-114].

S. aureus utilizes multiple strategies to evade IgG-mediated immune functions. S. *aureus* protein A is a cell-wall protein that binds IgG via the Fc region, thereby affecting its ability to act as an opsonin [71]. Some *S. aureus* isolates also modifies humoral host defenses by cleaving IgG that has bound to the bacterial surface, thereby inhibiting opsonophagocytosis [115]. Recently it was shown that staphylococcal proteases are responsible for the IgG cleavage. Serine protease inhibitors and cysteine protease inhibitors decreased the cleavage of surface-bound IgG resulting in increased complement activation and increased phagocytosis efficiency [116].

It is not clear whether the IgG response to *S. aureus* is sufficiently protective. As was recently shown using a set of eight *S. aureus* antigens, patients with a fatal course of *S. aureus* bacteremia had lower initial serum antibody concentrations than patients who recovered [117]. However, there was a pronounced heterogeneity in antibody levels against 19 *S. aureus* antigens among both healthy individuals and patients with different *S. aureus* infections [26]. In a different study, the antibody response following *S. aureus* bacteremia was followed in fifteen bacteremic patients and *S. aureus* isolates were typed by PFGE. Genes encoding 19 staphylococcal proteins were detected by PCR and the levels of IgG, IgM and IgA for those antigens were determined. It was found that isolates of *S. aureus* were not genetically related and each bacteremic

patient developed a unique immune response directed to different staphylococcal proteins [86].

Although *S. aureus* has not traditionally been considered an intracellular pathogen, its ability to enter and survive within phagocytic and non-phagocytic cells is now well established [118-121]. Intracellular survival as small colony variants may contribute to persistence of *S. aureus* in certain pathological conditions, such as endocarditis, osteomyelitis, and bovine mastitis [122]. Internalization and survival of *S. aureus* may explain why the humoral immune response alone is inefficient and why antibiotic treatment fails to eliminate this pathogen, which is well adapted to its host.

The immune response to *S. pseudintermedius* is largely an unexplored area, knowing the nature of the protective immune response to this pathogen may facilitate the design of new therapeutics especially after the emergence of antibiotic resistance. The objectives of this study are to examine the humoral immune response to *S. pseudintermedius* and to determine the patterns of antigen recognition by IgG from dogs with or without pyoderma. Levels of serum IgG antibody activity against *S. pseudintermedius* isolates of different genetic backgrounds (ST 68, ST 71, ST 26, ST 96 and ST 103) were determined with sera from canine healthy controls and patients with pyoderma. Serum IgG was tested for reactivity with antigens extracted from the following staphylococcal species*: S. aureus, S. intermedius, S. delphini*, S. *schleiferi* subsp*. schleiferi, S. schleiferi* subsp. *coagulans* and from S. *pseudintermedius* isolates of different sequence types. *S. pseudintermedius* isolates were examined for evidence of IgG protease and protein A activity.

Materials and Methods

Bacterial isolates and antigen preparation

Clinical isolates of *S. aureus*, *S. delphini, S. schleiferi* subsp. *schleiferi, S. schleiferi* subsp. *coagulans*, and *S. pseudintermedius* from dogs and a reference strain of *S. intermedius* were used in this study*. S. pseudintermedius* isolates were of the following sequence types: ST 68, ST 26, ST 71, ST 96, and ST 103 as previously characterized using MLST-8 (unpublished data) [7]. Bacterial isolates were clonally purified by streaking on Columbia Agar (BD, Sparks, MD) containing 5% sterile defibrinated sheep blood (Hemostat, CA). A single, well-isolated colony from each isolate was inoculated into 5 ml of Nutrient Broth

(Difco, France) and incubated at 37˚ in a rotary shaker at 220 rpm. Whole cell lysates were prepared from overnight cultures. The broth cultures were centrifuged at 10,000g for 6 m. The supernatant was discarded and the pellets were washed twice in sterile phosphate buffered saline PBS (pH 7.2). The pellets were suspended in an equal volume of sterile PBS. An equal volume of sample lysis buffer (4% SDS, 20% glycerol, 10% 2- mercaptoethanol, 0.004% bromophenol blue. pH 6.8, Sigma, Germany) was added, mixed and incubated at 100°C for 5 m. The tubes were centrifuged at 10,000g for 10 m. The supernatants were separated into new tubes and used for electrophoresis.

Study Population

Fourteen client-owned dogs with superficial pyoderma diagnosed by the University of Tennessee veterinary dermatology service were enrolled in this study. Dogs were excluded from the study if they were receiving immunosuppressive or immunomodulatory medications (including corticosteroids, cyclosporine, or cytotoxic agents), if they were systemically ill, or if they had a confirmed or suspected diagnosis of neoplasia or an uncontrolled endocrinopathy (including hypothyroidism and hyperadrenocorticism). Superficial pyoderma was diagnosed by compatible clinical lesions (papules, pustules, crusts, and/or epidermal collarettes) and skin cytology findings (inflammatory cells, intra- and/or extracellular bacterial cocci). Skin cultures were also performed from clinical lesions for isolation of causative organisms and susceptibility testing. A full physical examination was performed on each dog and the extent of the skin lesions was noted and recorded. Dogs with skin lesions affecting less than 25% of a single body area were noted as having localized pyoderma, while those with skin lesions affecting >25% of a single body area or multiple body areas were noted as having generalized pyoderma. For each affected dog, the primary disease diagnosis was recorded, as well as any medications (other than routine flea and heartworm preventives) that the dog was receiving at the time of diagnosis and whether the pyoderma was recurrent (at least one previous known infection). All dogs with pyoderma were treated with topical and/or systemic antimicrobials prior to the second examination; all treatments were also recorded.

Sixteen healthy control dogs, owned by students, faculty, and staff of the University Of Tennessee College Of Veterinary Medicine, were also enrolled in the study. Control dogs had no history of dermatologic or systemic disease,

normal physical examinations, and were not receiving medications other than routine flea and heartworm preventives.

For all dogs, two blood samples of 4 mls each were collected by jugular venipuncture. For affected dogs, the first blood sample was collected at the time of diagnosis. The owners were asked to return with their dog in one month's time, following antimicrobial therapy in dogs with pyoderma, for collection of the second blood sample. At this time, the affected dogs were examined by a member of the dermatology service for clinical and cytologic confirmation of pyoderma resolution. For all blood samples, sera was separated and frozen until used.
All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee.

Determination of the immune response

Flow cytometry analysis

An overnight culture was prepared by inoculation of 5 ml nutrient broth (Difco, France) with a single colony of *S. aureus*, *S. delphini, S. Schleiferi coagulans, S. Schleiferi, S. pseudintermedius* isolates belonging to ST 68, ST 71, ST 26, ST 96 ST, ST 103 and *S. intermedius* (ATCC 29663). Bacteria were centrifuged and pellets were washed with 5 ml PBS. After centrifugation bacteria were resuspended in flow buffer (3 ml fetal bovine serum, 60 ml of 0.5% sodium azide and 87 ml of PBS) and adjusted to optical density of 2.6 (+- 0.1 OD) at wavelength 550 nm. One ml of the suspension was mixed with diluted serum samples (1:100) and incubated for 1 hour at 4°C. The bacterial cultures were washed with 5 ml PBS and the pellets were suspended in affinity purified FITC conjugated anti-dog immunoglobulin G (Bethyl, USA) diluted 1: 500 in PBS and incubated for one hour at 4°C. The bacteria were washed with 5 ml PBS and the pellets were suspended in 1 ml PBS for analysis. A Beckman Coulter Epics XL flow cytometer with EXPO 32 software was used.
To determine if there was any difference between cultures in logarithmic and stationary phases, bacteria were cultured in nutrient broth and incubated in a shaking incubator for 5 hours and overnight. Bacteria were centrifuged and pellets were washed with 5 ml PBS. Bacteria were resuspended in flow buffer and adjusted to an optical density of 2.6 at wavelength 550 nm.

Western blot analysis

Western blots were used to assess the reactivity of antibodies with antigen extracts of various bacterial species. The whole cell lysates of each clinical isolate were resolved by running in SDS-PAGE in 10% polyacrylamide gels (Bio Rad, USA) with running buffer containing 0.025 M Tris, 0.192 M Glycine and 0.1% SDS. Proteins were electrophoretically transferred onto nitrocellulose membrane (NC) (Thermo scientific, USA). The transfer was carried out under standard conditions, and the resulting blot blocked overnight with 5% (w/v) nonfat dried milk powder in phosphate buffered saline containing Tween-20 (PBS-T - PBS, 0.1% Tween 20, pH 7.6) at 4°C. Western transfer buffer contained 0.025M Tris, 0.192M Glycine, and 20 % (v/v) methanol. The membranes were washed three times for 10 min each using PBS-T at 25°C. The blocked membranes were incubated with serum (diluted 1:100) in milk (PBS-T) for 60 min on a horizontal rotatory shaker at room temperature (RT). Membranes were washed three times as described above. Horseradish peroxidase-conjugated goat anti-dog immunoglobulin G secondary antibody (Bethyl, USA) was used at a dilution of 1:500 in milk PBS-T for 60 min at RT, followed by three washes as described above. The reactivity was visualized by exposure to 4- chloro-1-naphthol substrate (Thermo scientific, USA). The blots were dried and photographed. Western blotting was performed in 10 separate experiments using 5 sera from each group (pyoderma and healthy control).

For characterization of the serological response to antigens from each bacterial species whole cell lysates of the bacterium were resolved by SDS-PAGE in 10% polyacrylamide preparative gels and transferred onto NC membrane. After transfer, the NC membrane was cut vertically into strips, which were washed twice in PBS and soaked in blocking buffer containing 5% (w/v) nonfat dried milk powder in PBS- T overnight at 4ºC. Sera were diluted 1:100 in milk PBS-T and added to individual strips. After incubation for an hour at RT in horizontal rotatory shaker, the strips were washed with three changes of PBS-T for 10 m each. Horseradish peroxidase-conjugated goat anti-dog immunoglobulin G secondary antibody (Bethyl, USA) was diluted 1: 500 in milk PBS-T was added to each strip and incubated for another hour at 37ºC. Following three washings for 10 min each. The reactivity of the strips was developed by soaking for 5 m in substrate containing 4- chloro-1-naphthol. The blots were dried and photographed.

ELISA

Overnight cultures were prepared by inoculation of a single colony in 50 mL of sterile tryptic-soy broth (Becton Dickinson, Germany) for each isolate. The broth was incubated at 37°C for 24 hours. The cultures were then centrifuged at 4000 rpm for 10 minutes, 20 mL of PBS were added to the pellets and the pelleted bacteria were vortexed to resuspended and wash. The bacterial tubes were centrifuged at 4000 rpm for 10 minutes, the pellets were then suspended in carbonate/bicarbonate buffer (0.2 M Sodium carbonate bicarbonate, pH = 9.4), to an optical density of 1.0 at 550 nm. Hundred uL were pipetted into each well of a 96 well microtiter plate for coating. The plate was placed at 4°C overnight. The plate was washed three times with a plate washer (ELx 405 Biotech Instrument, USA) using PBS-T. A 1:100 dilution of serum in PBS-T (from patients or control animals) was prepared and 200 uL were added to each well. Serum from each time point was run in duplicate, and two control wells were left blank. Serial two-fold dilutions were prepared up to 1:800, and the ELISA plate was then placed in a 37°C incubator for 1 hour. The plate was again washed in the plate washer using PBS-T. Horseradish peroxidase-conjugated goat anti-dog immunoglobulin G secondary antibody (Bethyl, USA) was used at a dilution of 1:500 in PBS-T and 100 uL were added to each well including the two control wells. The plate was then incubated for 1 hour at 37°C. The plate was again washed using PBS-T. Hundred uL of TMB (3,3',5,5'-tetramethylbenzidine) was then added to each well.. After 5 minutes, the reaction was stopped using 100 uL per well of stop solution (0.18 M sulfuric acid). The absorbance at 450 nm was measured, using a plate reader (ELx 800 Biotech instrument, USA).

Determination of expression pattern of proteases and their effect on the IgG response

S. pseudintermedius was inoculated into tubes containing 5 ml of nutrient broth. Cultures were incubated for 5 hours and overnight at 37°C (for logarithmic and stationary phase of growth, respectively) and bacteria were pelleted. Supernatants were collected and concentrated by using Microcone filter cut-off size 3,000 daltons (Millipore, USA). The filters were centrifuged for 5 min at 3000 rpm, The concentrated supernatant was washed 2 times with 2ml PBS and centrifuged for 2 min at 1000 rpm. To determine if the concentrated *S. pseudintermedius* supernatants possessed protease activity, 2 techniques were used:

1- Quanti-Cleave protease assay kit (Thermo scientific, USA) was used. Procedures were done according to the manufacturer's instructions, briefly, 100 µl of succinated casein were placed in a set of wells in a microtiter plate (unknown samples and positive control), and 100 µl of assay buffer were placed in blank wells. 50 µl of supernatants were added to the sample and the corresponding blank well. 50 µl of trypsin at a concentration of 0.5 mg/ml were added to a set of wells to serve as a positive control. The plate was incubated at 37°C for 20 min. 50 µl of the supplied BSAM working solution were added to each single well. The plate was incubated at 37°C for 20 min. The color absorbance was read at 450 nm.

2- Zymogram SDS

Procedures were done according to the manufacturer's instructions (Bio-Rad, USA). Briefly, one part of the sample was mixed with two parts of Zymogram sample buffer, after vortexing, 20 µl of each sample and trypsin (positive control) were applied and electrophoresed in 1 x Tris-Glycine SDS running buffer according to standard running conditions. After running the gel was incubated with 1x Zymogram renaturing buffer with gentle agitation for 30 minutes at RT. Zymogram renaturing buffer was decanted and equilibrated with 1x Zymogram developing buffer for 30 minutes at RT then replaced with fresh 1x Zymogram developing buffer and incubated for at least 4 hours at 37°C. The gel was stained with 0.5% comassi blue (w/v in 40% methanol and 10% acetic acid) for 1 hour. Gels were destained with 40% methanol, 10% acetic acid and 50% water. Gels were examined for zones of lysis around samples as an indication of protease activity.

Determination of putative protein A activity in *S. pseudintermedius*

S. pseudintermedius isolates ST 68, ST 71, ST 26, ST 96 and *spa* positive *S. aureus* isolate (ATCC 12598) were cultured in nutrient broth overnight at 37°C. Bacteria were centrifuged and pellets were washed with 5 ml PBS. After centrifugation bacteria were resuspended in flow buffer and adjusted to optical density of 2.6 (+- 0.1 OD) at wavelength 550 nm. The bacterial pellets were mixed with 100 µl of 10 fold serial dilutions of canine IgG (Innovative, USA). The samples were incubated at 37°C for 30 minutes. Pellets were washed with 5 ml PBS. After centrifugation, the pellets were suspended in 25 µl affinity purified

FITC conjugated anti-dog immunoglobulin G (Bethyl, USA) diluted 1: 500 in PBS and incubated for one hour at 4°C. The mix was washed with 5 ml PBS and the pellets were suspended in 1 ml PBS for analysis. The experiment was repeated 3 times.

Statistical analysis

Flow cytometry and ELISA data were analyzed as a three factor ANOVA: one fixed factor (treatment) and two within-subjects factors (time and antigen type). The effect of the type of antigens, time and treatment were assessed.

Results

Affected and Control Dog Demographics

Dogs with pyoderma represented 7 different breeds and mixed breeds. The breed make-up was as follows: mixed breed, 4/14 (29%); Labrador Retriever, 3/14 (21%); Dachshund, 2/14 (14%); Golden Retriever, 1/14 (7%), Boykin Spaniel, 1/14 (7%), Miniature Schnauzer, 1/14 (7%) Havanese, 1/14 (7%), Cocker Spaniel, 1/14 (7%). Ten of 14 (71%) of dogs with pyoderma were spayed females, while 2/14 (14%) each were castrated males and intact females, respectively. Affected dogs ranged in age from 2 years to 12 years, with a median age of 5 years and a mean age of 5.8 years. The most common primary disease diagnosis was allergic dermatitis (including flea allergy dermatitis, atopic dermatitis, or cutaneous adverse reaction to food), representing 13/14 (93%) dogs, while one dog had an unknown primary disease. One of the 14 dogs had concurrent diagnoses of flea allergy dermatitis and generalized demodicosis, while 2/14 dogs had concurrent diagnosises of allergic dermatitis and well-controlled hypothyroidism.

Twelve of 14 (86%) dogs were diagnosed with generalized superficial pyoderma, while 2/14 (14%) were diagnosed with localized superficial pyoderma. Methicillin susceptible *S. pseudintermedius* (MSSP) was isolated on skin culture from 11/14 (79%) dogs, while methicillin resistant *S. pseudintermedius* was isolated from the skin lesions of 2/14 (14%) dogs. Mixed isolates of MSSP, methicillin susceptible *S. schleiferi* subsp. *coagulans*, and *Pseudomonas aeruginosa* were isolated from

skin lesions of one dog. Two of 14 (14%) affected dogs were treated with systemic antimicrobials alone, while 2/14 (14%) dogs (both with localized pyoderma) were treated with topical antimicrobials alone, and 11/14 (79%) were treated with a combination of topical and systemic antimicrobial therapy. Affected dogs were re-examined and the second blood sample was drawn 21-59 days following the initial examination, with a mean of 37 days between the first and second blood collections. The pyoderma was clinically resolved in 9/14 (64%) dogs at the second time point and was unresolved in 5/14 (36%) dogs. In two of these 5 dogs (40%), MRSP was isolated on skin culture. One of 5 dogs (20%) with unresolved pyoderma at re-examination was initially diagnosed with localized infection; all other dogs with unresolved lesions had generalized pyoderma. Ten of 14 (71%) dogs had recurrent pyoderma, while 4/14 (29%) did not. In four of 5 (80%) dogs with unresolved pyoderma, the pyoderma was also recurrent.

Healthy control dogs represented 6 different breeds and mixed breed dogs. The breed make-up was as follows: mixed breed, 8/16 (50%); Pomeranian, 2/16 (12.5%); Australian Cattle Dog, 2/16 (12.5%); Dachshund, 1/16 (6%); Cocker Spaniel, 1/16 (6%); Border Collie, 1/16 (6%); Labrador Retriever, 1/16 (6%). Ten of 16 (62.5%) control dogs were castrated males, while 6/16 (37.5%) were spayed females. Healthy dogs ranged in age from 1 year to 12 years, with a median age of 6 years and a mean age of 5.6 years. The second blood sample was drawn from healthy dogs 29-63 days following the initial sampling, with a mean of 38 days between the first and second blood samplings.

IgG reactivity to *S. pseudintermedius* antigens

Flow cytometry

The three way- interactions (treatment, time and antigen types) were significant, by splitting off the three factors interactions: there was a significant difference between antigen types in the patient group and time had an effect. Table 3.1 shows how the pattern of mean differences between antigen types differed between time 1 and time 2 within the patient group. ST 96 had the highest arithmetic mean which was not significantly different from ST 71. In the control group, there was no effect of time. The only difference was the antigen type, the highest reactivity was found in ST 96 followed by *S. intermedius*, ST 71 and *S. aureus* (Table 3.2)

S. aureus isolate (ATCC 12598) served as a positive control for the detection of putative *spa*-like receptor mediated binding of IgG by one or more functionally similar protein in *S. pseudintermedius.* There was no obvious activity for putative protein A in *S. pseudintermedius* (Figure 3.1)

Table 3.1 Reactivity of canine IgG with staphylococcal antigens in the patient group as determined using flow cytometry.

TIME	Type of antigen	N. of Observation	Mean	Std Dev
1	ST68	14	29.0000 DE*	12.1465
	ST 71	14	65.2857 AB	22.2691
	ST 26	14	58.3571 BC	29.8422
	ST 96	14	94.3571 A	43.0681
	ST 103	14	25.6429 E	15.3503
	S. aureus	14	42.0000 CD	25.5945
	S. intermedius	14	50.4286 BC	27.2643
	S. delphini	14	6.0000 G	6.3851
	S. sch. schleiferi	14	11.3571 F	6.4879
	S. sch. coagulans	14	44.6429 CD	28.5862
2	ST68	14	25.5000 CD	13.9931
	ST 71	14	39.5714 BC	26.1702
	ST 26	14	27.0714 CD	12.9167
	ST 96	14	51.0000 A	46.2551
	ST 103	14	37.0000 BC	17.2091
	S. aureus	14	36.1429 BC	13.8334
	S. intermedius	14	23.0714 D	16.657
	S. delphini	14	11.7857 E	5.632
	S. sch. schleiferi	14	10.9286 E	3.8323
	S. sch. coagulans	14	45.3571 AB	22.4589

*Means in the same letter group are not significantly different at P value of 0.05
In the first time point:
ST 96 was the highest reactive and ST 71 was the second most reacting ST with no significant difference between them. ST 26 and *S. intermedius* were highly reacting and they were not significantly different from ST 71. *S. sch. coagulans* and *S. aureus* were not significantly different from ST 26 and *S. intermedius*. ST 68 was not significantly different from S. *aureus* and *S. sch. coagulans*. ST 103,

S. sch. schleiferi and *S. delphini* were the least reactive respectively and they were significantly different from each other. In the second time point: Reactivity in the second time point was significantly different from the reactivity in the first time point. ST 96 was the highest reactive followed by *S. sch. coagulans* and there were no significant difference. ST 71, ST 103 and *S. aureus* were highly reactive and not significantly different from each other and from *S. sch. coagulans. S. sch. Schleiferi* and *S. delphini* were the least reactive respectively and they were not significantly different from each other.

Table 3.2: Reactivity of canine IgG with staphylococcal antigens in the control group as determined using flow cytometry.

Type	N. of Observation	Mean	Std Dev
ST68	14	22.2500 D*	25.3999
ST 71	14	37.3571 B	20.2379
ST 26	14	25.4815 CD	25.7926
ST 96	14	63.9286 A	47.7089
ST 103	14	21.6429 DE	20.3401
S. aureus	14	30.9643 BC	7.1827
S. intermedius	14	44.6786 B	32.8262
S. delphini	14	12.2143 F	5.7113
S. sch. schleiferi	14	14.1429 EF	12.2526
S. sch. coagulans	14	31.4643 BC	16.8512

*Means in the same letter group are not significantly different at P value of 0.05 The highest reactive ST was ST 96 and *S. intermedius* was the second most reactive species, there was a significant difference between them. There was no significant difference between ST 71, *S. sch.* subsp*. coagulans, S. aureus* and *S. intermedius.* There was no significant difference between ST 68, ST 26 and ST 103. The lowest reactive species were *S. sch.* subsp *schleiferi* and *S. delphini*

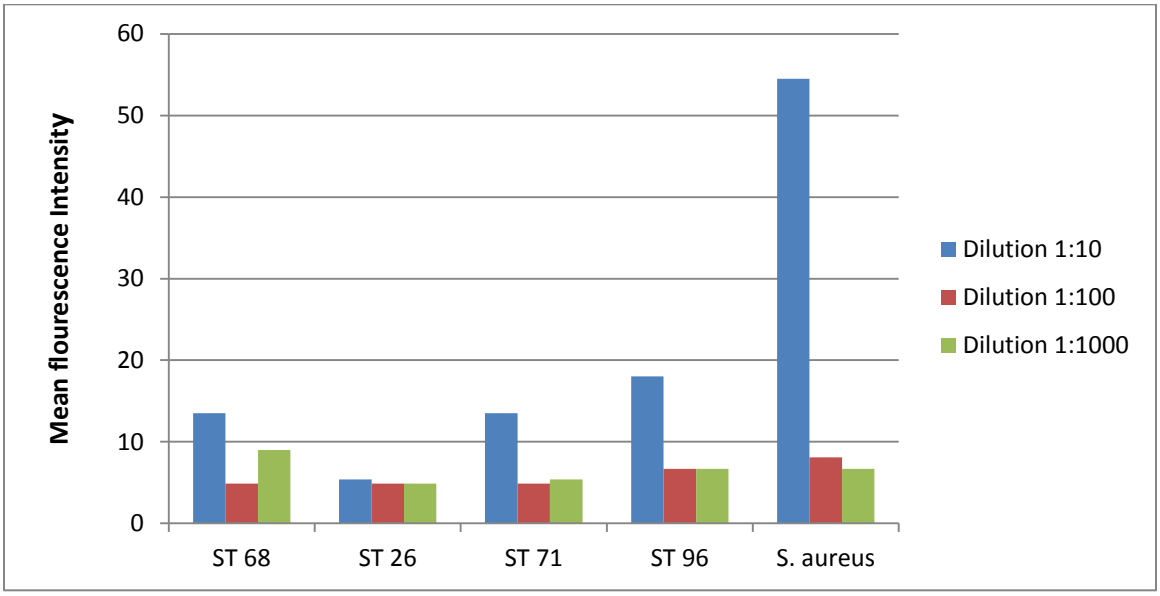

Figure 3.1:
Protean A putative activity of ST 68, ST 26, ST 71, ST 96 and *spa* positive *S. aureus* isolates as determined by flow cytometry.

Western blot

Fourteen *S. pseudintermedius* pyoderma patient sera were reacted against whole-cell antigens prepared from different genetic backgrounds (ST 68, ST 71, ST 26, ST 96 and ST 103). There was a common set of bands consisting of at least 6 immunoreactive antigens ranging from 200 to 20 KD in the 5 genetic backgrounds (table 3.3). At least three of them (ranging between 76 to 30 KD) were found in sera from both infected and control groups. There were proteins reacted with sera in patient group (ranging between 200 to 70 KD) as shown in figure 3.2. They were not reacted with sera in the control group.

Table 3.3: Number and molecular weight (KDa) of protein bands of the five different *S. pseudintermedius* isolates and the other staphylococcal species

	ST 68	ST 71	ST 26	ST 96*	ST 103	*S. intermedius*	*S. aureus*	*S. sch. schleiferi*	*S. sch. Coagulans*
Average Number of bands	14	15	15	17	16	9	6	5	3
Mt Wt. range of bands	20-200	20-200	20-200	20-200	20-200	200-30	50-150	70-30	70-30
Mt Wt. of most intense band	102	102	102	102	60	110	80	40	52
Mt Wt. of 2nd most intense band	30	30	200	60	102	60	150	55	40
Mt Wt. of 3rd most intense band	200	35	70	70	200	70	56	62	70

*ST 96 and ST 103 had the largest number of protein bands

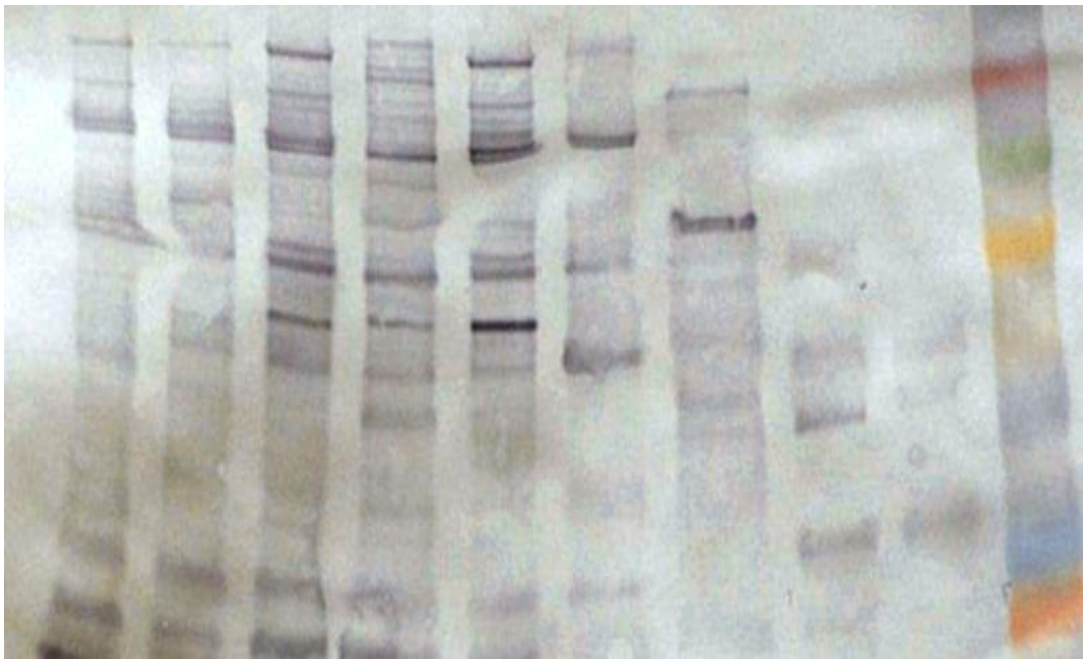

Figure 3.2. Banding patterns of *S. pseudintermedius* STs and other staphylococcal species against patient serum

(1) ST 68, (2) St 71, (3) ST 26, (4) ST 96, (5) ST 103, (6) *S. intermedius*, (7) *S. aureus*, (8) *S. schleiferi coagulans* and (9) *S. schleiferi* subsp. *schleiferi* were separated on 10 % poly acrylamide gels and electroblotted onto NC membranes. Membranes were probed with pyoderma serum and goat anti-dog immunoglobulin G- horseradish peroxidase HRP conjugate and the colors were developed with the substrate

ELISA

Using antigens prepared from different *S. pseudintermedius* sequence types (ST 96, ST 68, ST 71) and *S. schleiferi* subsp. *schleiferi*, the three way- interactions (treatment, time and antigen types) were non-significant; however the treatment by time and the treatment by antigen type interactions were significant. Time had no effect in the control group (T1= 0.7439, T2= 0.7612). In the patient group, regardless of antigen type, there was a significant difference between time 1 and time 2 (T1= 0.9252, T2= 0.8603). In the control group there was no significant difference between ST 103, ST 68 and ST 71; however, there was a significant difference between *S. sch. schleiferi* antigens and each of the *S. pseudintermedius* antigens as shown in table 3.4. In the patient group, there was a significant difference between all of the antigen types as shown in table 3.3 and time had an effect. The highest reactivity was found in ST 71 (T=1.364) followed by ST 103 (T= 1.04225) and ST 68 was 0.7388.

IgG reactivity to other Staphylococcal antigens

For *S. intermedius* there were at least 2 immunoreactive antigens (110 and 70 KD) shared between the 2 species. For *S. aureus,* There was one main immunoreactive antigen (80 KD) (figure 3.2, table 3.3).
S. schleiferi subsp. *coagulans* immunoreactive antigens were ranging between76 to 30 KD and two of them were shared with *S. pseudintermedius* antigens. Antigens prepared from *S. schleiferi* subsp. *schleiferi and S. delphini* were not reactive with both the healthy and pyoderma sera. The results were confirmed with flow cytometry. *S. intermedius* was the most reactive staphylococcal species in the control group and *S. aureus* was the most reactive species in the patient group. *S. schleiferi* subsp. *coagulans* was the next most reactive species in both the groups (table 3.1 and table 3.2)

Table 3.4: Reactivity of canine IgG with staphylococcal antigens in the two time points as determined using ELISA

Treatment	type	Time	N. of Observation	Mean	Std Dev
Control	ST103	1	14	0.8345 A*	0.2835
		2	14	0.8873 A	0.3587
	ST68	1	14	0.7638 A	0.3263
		2	14	0.7555 A	0.3523
	ST71	1	14	0.9030 A	0.261
		2	14	0.9334 A	0.2541
	S. sch. schleiferi	1	14	0.4742 B	0.2353
		2	14	0.4687 B	0.2578
Patient	ST103	1	14	1.0664 C	0.4451
		2	14	1.0181 D	0.4202
	ST68	1	14	0.7646 E	0.2503
		2	14	0.713 F	0.2067
	ST71	1	14	1.4152 G	0.3837
		2	14	1.3146 H	0.3533
	S. sch. schleiferi	1	14	0.4546 I	0.1998
		2	14	0.3955 J	0.1568

*Means in the same letter group are not significantly different at P value of 0.05 There was no significant difference between ST 103, ST 71 and ST 68 in the control group and they were significantly differed from *S. sch. schleiferi*. In the patient group, there was a significant difference between all the antigens. The highest reacting one was ST 71 and the lowest was *S. sch. schleiferi*

Discussion

The immune response to *S. pseudintermedius* is poorly understood. Elucidating the protective immune response is a fundamental step toward the development of immune-therapies to treat and protect from staphylococcal infections. Staphylococcal species have complex genomes with heterogeneity in gene expression patterns especially among those that encode surface proteins which lead to differences in the immune response. That is why some specific genetic backgrounds (versus expendable virulence genes are linked to certain diseases [72, 123]. There was a heterogeneous antibody response against *S. aureus* in bacteremia patients which was related to genetic differences in *S. aureus* isolates [124]. This heterogeneity suggests that a protective vaccine should be based on multiple components.

The IgG response of 14 dogs with pyoderma and 14 dogs with no history of pyoderma to staphylococcal proteins extracted from 5 genetically different *S. pseudintermedius* isolates were studied using three techniques, flow cytometry, ELISA and western blot. There were differences in the IgG reactivity between the different genetic backgrounds as revealed by the antigen banding pattern in western blots. A similar study was published in 2008 [125], antigens were extracted from 6 *S. intermedius* isolates and were reacted with sera from 21 dogs with various skin diseases. Homogeneity in the protein banding patterns in both comassi staining and western blot was found between the 6 isolates. The 6 isolates were isolated from cases of pyoderma and otitis media. They were identified biochemically and no further genetic characterization was made. These isolates were most probably *S. pseudintermedius* belonging to the same ST. A significantly different reactivity between the 5 isolates was found using flow cytometry and ELISA. The highest reactivity was found in ST 96 and ST 71. ST 68 and ST 103 were the lowest reactive STs of *S. pseudintermedius.* ST 96 was described in a susceptible isolate in the USA and it has the closest genetic background to ST 68, the major methicillin resistant clone in the USA. They differ in 3 out of the 8 genes of MLST-8. The difference in reactivity between ST 96 and ST 68 emphasizes the correlation of genetic background and antibody reactivity.

Major differences were found in the IgG response to the other staphylococcal species. Reactivity of sera to *S. aureus* antigens was moderate. Although colonization with *S. aureus* seems to be uncommon among healthy dogs, some recent studies highlight the possibility of the transmission of *S. aureus* between

human to dogs [126-129]. Of those, a report showed high prevalence of the isolation of MRSA from dogs with household colonized people. The dog isolates were indistinguishable from the human isolates suggesting that interspecies transmission of MRSA is possible [126]. There is also a possibility of antibody to *S. pseudintermedius* cross-reacting with *S. aureus* antigens. Some reports described surface proteins in *S. pseudintermedius* with high similarity to those in *S. aureus [81].*

S. *intermedius* was the highest reacting staphylococcal species after *S*. *pseudintermedius* in the control group and it is the closest genome to *S. pseudintermedius [10]*. This reactivity might be due to the expression of similar antigens cross-reacting with *S. pseudintermedius* antibodies. S. *schleiferi coagulans* was the highest reacting staphylococcal species in the patient group after *S. pseudintermedius* and no difference was noted between the first and the second blood sample reactivity. This reactivity may be due to previous exposure to this bacterium in some of the dogs as it had been isolated from the external auditory meatus of dogs suffering from external otitis [130, 131]. Although they were reported as phylogeneticaly close species [10], *S. delphini* is the lowest reactive among the staphylococcal species.

The IgG response to *S. pseudintermedius* seems to correlate with the outcome of infection. The pyoderma infection in five of the fourteen patients was not resolved at the second blood sample. In those five patients, the IgG level in the second time point was lower than the IgG in the first blood sample. In all of the other patients, having the same or higher level of IgG response was correlated with the resolution of the infection.

Factors associated with the involvement of *S. pseudintermedius* in infections are not fully clear. The ability of *S. pseudintermedius* to adhere to canine skin is likely to be an important factor in the initiation of infection. S. *pseudintermedius* strains were shown to adhere to immobilized fibrinogen, fibronectin and cytokeratin 10 [81]. They can secrete α, β and γ haemolysins, protease, coagulase, enterotoxin and exfoliative toxin [23, 24]. Comparing healthy control to pyoderma patients in terms of antibodies against staphylococcal proteins, there was a common signature consisting of three low molecular weight proteins expressed constitutively regardless of infection. These proteins are considered important however, information about whether they are surface exposed is needed. There were four proteins expressed only in pyoderma patients including 180 KD protein

which may be the same protein which reacted with antibodies to staphylococcal fibrinogen-binding proteins *sdrD* and *cifA* reported previously [81].

S. pseudintermedius may differ from *S. aureus* in some of the mechanisms of evasion of the immune system. *S. aureus* Protein A (*spa*) has a molecular weight of 42 KDa and is able to bind non-specifically to the Fc fragment of IgG of several species, including dogs [71]. An equivalent to *spa* was described in the genome of *S. pseudintermedius* (IgG-binding protein) and used for developing a species-specific *spa* typing protocol [45]. Our results indicated the lack of the activity of this gene in ST 68 isolates compared to *spa* positive *S. aureus*. *S. aureus* also express proteases from the serine type and their role is the degradation of the major opsonins: human IgG and human C3b [115]. There is no evidence for this activity in *S. pseudintermedius.*

In this study the immune response to different genetic backgrounds of *S. pseudintermedius* was determined using a uniform group of *S. pseudintermedius* pyoderma patients and a group of healthy controls. The results indicated the heterogeneity of the immune response to the various genetic backgrounds in both the groups. There was no evidence for proteases activity or *spa* like activity in *S. pseudintermedius*. More studies are recommended to examine the role of other immune evasion mechanisms on the IgG response.

Chapter 4: Conclusions and Application of Findings

Understanding the genomic diversity is a fundamental step toward understanding the immune response to a bacterium and the identification of potential vaccine targets. *S. pseudintermedius* occurs as clonal populations the members of which likely share surface antigens. *S. pseudintermedius* was shown recently with high prevalence of methicillin resistance and other antibiotics resistance which limit the drug choices to treat *S. pseudintermedius* infections. In the not too distant future there may be few antibiotics to treat such infections. Because of that designing a vaccine is a high research priority.

The development of MLST-8 has provided a tool to elucidate the population genetics in *Staphylococcus pseudintermedius.* By using MLST-8, 106 STs were analyzed which indicated a high level of genetic diversity of the species which is not surprising for a staphylococcal species [132, 133]. Most of this diversity, however, is limited to methicillin susceptible isolates. Methicillin resistant isolates, on the other hand, are represented by a limited number of highly successful clonal types which have apparently spread over wide geographic regions. Once MLST-8 is put to use by the *S. pseudintermedius* research community an even better understanding of the clonal nature of *S. pseudintermedius* and its spatial characteristics and population genetics should emerge.

By choosing 5 distinct genetic backgrounds to measure their reactivity against pyoderma dog sera and healthy control sera, variations in the immune response were found even between two genetically linked STs, ST 68 and ST 96. They both are USA isolates and they differ in 3 out of 8 loci. The difference in the intensity of the reactivity of the antigens that reacted was significant and the reactivity of ST 96 was the highest among *S. pseudintermedius* and the other staphylococcal species. The variation in surface reactivity and banding patterns between different genetic backgrounds of *S. pseudintermedius* suggest that a vaccine for *S. pseudintermedius* may be challenging. Further work, however, needs to be done to determine if conserved antigens occur and are shared by proteins with different sizes. The ideal candidate would be a conserved surface protein, expressed across the different genetic backgrounds and evoke protective antibodies.

The differences in antibody reactivity with *S. pseudintermedius* proteins between infected and non- infected dogs were primarily high molecular weight proteins

recognized by infected dogs. Although the identity and function of the proteins is unknown they might be virulence factors or immune evasion mediators which facilitated clinical disease. For all dogs there was a common set of low molecular weight proteins. This is encouraging as they may represent conserved antigens which may be suitable as vaccine candidates. Additional testing would be required to determine if they are surface exposed.

The reactivity of antigens prepared from different species within the *Staphylococcus intermedius* group (SIG) with dog sera showed that although there are minor differences between the genomes of the three members there are major differences in the immune reactivity especially between *S. pseudintermedius* or *S. intermedius* and *S. delphini.* The former species was highly reacting whereas the later was the lowest reacting. Since *S. intermedius* and *S. delphini* are not associated with canine infections it is likely that the antibody reactivity with these species was a cross-reaction with *S. pseudintermedius* immunogens.

In 2010 McCarthy and Lindsay studied the relationship between surface and immune evasion gene variation and genetic backgrounds in *S. aureus*. They demonstrated that variation in genes encoding surface proteins and genes encoding secreted proteins predicted to interact with host immune responses is lineage specific. Most of the variations occurred in predicted functional domains and some surface proteins were missed or truncated in some lineages. Some domains were found to be conserved across the lineages [132] and they concluded that successful staphylococcal vaccines should contain cocktails of antigens representing all variants. Our work prepares a foundation for identifying representative members of the predominant lineages and candidate antigens to extend this concept for vaccine development to *S. pseudintermedius*.

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Appendix

Table 2.1.A. Allele description of *purA* gene

PurA	nucleotide position										
allele	105	113	174	186	192	258	339	342	367	396	399
1	T	T	C	G	C	C	T	G	G	T	G
2	T	T	C	T	T	C	T	G	G	T	G
3	T	T	C	T	T	T	C	A	G	C	G
4	T	T	C	T	T	C	T	G	G	C	G
5	C	T	C	G	T	C	T	G	G	C	G
6	T	T	C	G	T	C	T	G	G	C	G
7	T	T	C	G	C	T	T	G	G	C	G
8	C	T	C	G	C	T	T	G	G	C	G
9	T	T	C	G	C	C	T	G	G	C	T
10	C	T	C	G	C	C	T	G	G	C	G
11	T	T	C	T	T	C	T	G	A	C	G
12	T	T	C	G	C	C	C	A	G	C	G
13	T	T	C	G	C	C	T	G	G	C	G
14	T	T	C	G	T	T	C	G	G	C	G
15	T	C	C	G	C	T	T	G	G	C	G
16	C	T	T	G	C	T	T	G	G	T	G
17	T	T	C	T	T	T	T	A	G	C	G
18	C	T	C	G	T	C	T	G	G	T	G
19	C	T	C	G	C	T	T	G	G	T	G
20	C	T	C	G	T	T	T	G	G	C	G

Table 2.2.A. Allele description of *fdh* gene

fdh	nucleotide position				
allele	19	103	106	112	250
1	C	G	C	G	G
2	C	A	T	A	G
3	C	A	T	A	T
4	A	A	T	A	G
5	C	G	C	G	T

Table 2.3.A. Allele description of *pfoR* gene

pfoR	nucleotide position									
allele	51	72	87	93	99	126	281	342	354	372
1	T	T	G	A	G	G	C	A	T	G
2	T	T	G	A	G	G	C	C	C	G
3	T	T	G	A	A	G	C	A	T	G
4	T	T	A	A	G	G	C	C	C	G
5	T	T	G	A	A	G	C	C	T	G
6	T	T	G	G	G	G	C	A	T	A
7	T	T	G	A	G	G	C	C	T	G
8	T	C	G	A	G	G	C	C	C	G
9	T	T	G	A	G	A	C	A	T	G
10	T	T	G	A	A	G	C	C	C	G
11	T	T	G	G	G	G	A	A	T	G
12	T	T	G	A	A	A	C	C	T	G
13	C	T	G	A	A	G	C	C	T	G
14	T	T	A	A	G	G	C	A	T	G

Table 2.4.A. Allele description of *sar* gene

sar	nucleotide position							
allele	43	67	146	208	238	256	341	358
1	G	G	C	A	C	C	G	G
2	G	G	C	T	C	C	G	G
3	G	G	C	A	T	C	G	A
4	A	A	C	A	C	C	G	G
5	A	G	C	A	C	C	G	G
6	G	G	C	A	T	C	G	G
7	G	G	C	A	C	C	A	G
8	G	G	T	A	C	T	G	G
9	G	G	C	T	T	C	G	G
10	A	G	C	A	T	C	G	G

Table 2.5.A. Trimming sequences of the four new genes

Gene	Trimming sequences
purA	5'TAT GCA GCT GGT CAA CG 3'CGT CGT GTG AGY GGG AT
sar	5'GTT AAA AGG TGA CGA AC 3'GAT CAA CTC GTT CAT GA
pfoR	5'AAT GCG GCC ATC GCA CC 3'GGG CGA AAC ACC AAT GG
fdh	5'TTC AAT TGA ACC TGA AT 3'CCC TTT CAC ACA TGA AGA A

Table 2.6.A. The distribution of the alleles of the 4 new genes in 176 isolates

Allele	*purA*	*fdh*	*pfoR*	*sar*
Allele 1	80	88	63	117
Allele 2	9	57	28	41
Allele 3	9	12	38	2
Allele 4	21	18	10	8
Allele 5	15	1	8	5
Allele 6	2		18	1
Allele 7	8		1	1
Allele 8	12		3	1
Allele 9	1		2	
Allele 10	1		1	
Allele 11	1		1	
Allele 12	1		1	
Allele 13	7		1	
Allele 14	3		1	
Allele 15	1			
Allele 16	1			
Allele 17	1			
Allele 18	1			
Allele 19	1			
Allele 20	1			

Vita

Samar Mansour Solyman was born in November 7, 1979 in Ismailia, Egypt. In 2007 she married Hassan Hassan. She is the daughter of Mohammed Mansour Solyman and Fatma Solyman Amer. she earned her B.S. in pharmaceutical sciences in 2001 and Master degree in Microbiology and Immunology in 2005 from the University of Suez Canal. She received the Egyptian PhD scholarship in 2006 and is currently assigned to the department of Microbiology and Immunology- College of pharmacy- Suez Canal University. Merit is her daughter and Marwan is her son.